Development of L1210 mutants in NAD metabolism

Sujareerat, Charin

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DEVELOPMENT OF L1210 MUTANTS IN NAD METABOLISM

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CHARIN SUJAREERAT

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C. Sujareerat

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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ABSTRACT

Clones of L1210 cells have been isolated in the presence of toxic concentration of 5 mM 3-acetamidobenzamide (3-AAB), an inhibitor of ADPR-transferase. It was initially suggested that resistance to the drug could be due to a defect in the process of ADP-ribosylation, especially cells deficient in ADPR transferase. Results show however that mutant cell lines have ADPR transferase activity and its activity is completly inhibited by 3-AAB. Furthermore, 3-AAB also enhances cytotoxicity of DMS in mutant cell lines. Other characterizations of mutant cell lines indicate that they have prolonged generation times in the presence and absence of 5 mM 3-AAB. They are sensitive to 3-aminobenzamide and 3- nitrobenzamide which are ADPR transferase inhibitors. Mutant 3 however has shown to be more resistant to 3-aminobenzamide than wild-type cells. The characterization of ADPR transferase in mutant 3 has shown that the Km and Vmax values for NAD are 3 times higher than those obtained in wild-type cells and it has a higher DNA content (2 fold) compared with wild-type cells. Mutant 3 may be of value in other aspects not related to ADP-ribosylation. The usefulness of mutant cells can be verified when further investigation on the genetic abnormalities that contribute the resistance property is determined.

To

SINGTONG, SINGAO, GAOFA

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ABBREVIATIONS

3-AAB	3-Acetamidobenzamide
3-AABA	3-Acetamidobenzoic Acid
3-AB	3-Aminobenzamide
ADPR	Adenosine diphosphate ribose
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BUdR	5 - bromo-2-deoxyuridine
cpm	Counts per minute
DTT	Dithiothreitol
DMS	Dimethyl sulphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
sDNA	Small deoxyribonucleic Acid
EGTA	Ethylene glycol-bis (b-amino-ethyl-ether)-N, N-tetra-acetic acid
EMS	Ethylmethane sulphonate
GTP	Guanine triphosphate
HEPES	N-2-Hydroxyethyl-piperazine-N'-2-ethane sulphonic acid
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
HMG	High mobility group
HPLC	High pressure liquid chromatography
IU	International unit
Ki	Inhibition constant
MMS	Methylmethane sulphonate

MNNG	N'-Methyl-N'-nitro-N-nitrosoguanidine
Na	Nicotinic acid
NaAc	Sodium acetate
NaAD	Nicotinic adenine dinucleotide
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced form of nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NaMn	Nicotinic mononucleotide
3-NB	3-Nitrobenzamide
3-NBA	3-Nitrobenzoic Acid
Nm	Nicotinamide
NMN	Nicotinamide mononucleotide
PCA	Perchloric Acid
PBS	Phosphate buffered saline
PEI	Polyethyleneimine
Poly (ADP-ribose)	Poly (adenosine diphosphate ribose)
PPO	2,5 - Diphenyl - Oxazole
PR - AMP	2' (5" - phosphoribosyl) - 5' AMP
R_f	Relative mobility (Rate of flow) Retardation Factor
RNA	Ribonucleic Acid
TCA	Trichloroacetic Acid
TTP	Thiamine triphosphate
t.l.c.	Thin layer chromatography
UV	Ultra violet

CHAPTER 1

INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

ADP-ribosylation is a protein modification process which occurs widely in nature (see 1.1.2). NAD is used as a substrate and as part of a molecular mechanism for modulating the activity of a wide variety of cellular proteins (see 1.1.1). The reactions are catalysed by enzymes called ADPR transferases.

It has been about 25 years since the discovery of poly (ADP- ribose), a nuclear modification, by Chambon *et al.*,(1963). Protein ADP-ribosylation has also gained an increasing amount of attention which is attributable to its postulated role in the DNA repair process (see 1.1.9). Because of its proposed involvement in this and other major cellular events such as cell proliferation (see 1.1.6), differentiation (see 1.1.8), DNA metabolism (see 1.1.7) and gene expression (see 1.1.7), the poly (ADP-ribose) field is, therefore, attracting many workers, especially from established mammalian DNA repair laboratories.

The introduction of this thesis will give a general review of nuclear ADP-ribosylation with particular emphasis on some of the reports emerging during the last few years (Part I) together with a review of mammalian cell mutation (Part II). The overall aim of this thesis is the development of, and investigation into ADPR transferase mutants in a mouse L1210 cell line.

PART I

1.1.1 METABOLISM OF NAD

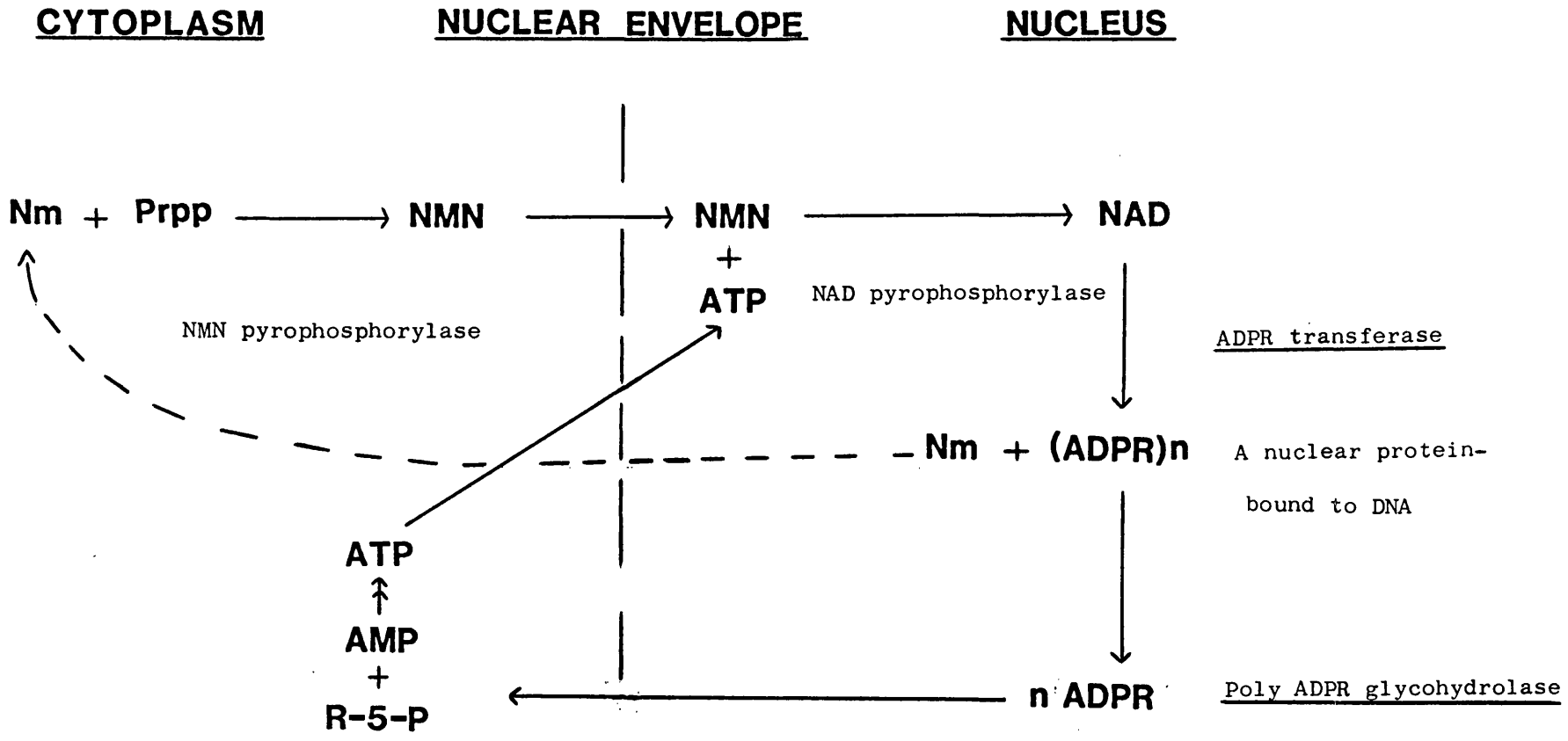
The process of NAD synthesis (see figure 1) starts in the cytoplasm. NMN is synthesized by NMN pyrophosphorylase from nicotinamide and phosphoribosyl pyrophosphate. NMN diffuses into the nucleus for the next step, where NMN and ATP are coupled to form NAD by NAD pyrophosphorylase. This enzyme has been found to be located exclusively in the cell nucleus (Hogboom and Schneider, 1952).

NAD is broken down in the cell in a variety of ways but the main pathway of breakdown (90%) occurs via the ADPR transferase systems (Rechsteiner *et al.*, 1976). About 5% is used in the normal coenzyme function of NAD in intermediary metabolism within the cell (Hillyard *et al.*, 1973). It has been shown that the half-life of NAD in human cell line D98/AH2 is about one hour (Reichsteiner *et al.*, 1976).

1.1.2 BIOSYNTHESIS AND BIOCHEMICAL CHARACTERIZATION OF POLY (ADP - RIBOSE)

Poly (ADP-ribose) is a unique biopolymer of nuclear origin, consisting of repeating units of ADP-ribose formed by the DNA bound enzyme ADPR transferase. This enzyme appears to be capable of catalyzing three separate related reactions (Ueda *et al.*, 1980) (figure 2).

1) Initiation (ADP-ribose) transfer to acceptor protein
2) Branching (addition of ADP-ribose) units on to poly (ADP- ribose) chains



Nm = Nicotinamide

Prpp = Phosphoribosyl pyrophosphate

NMN = Nicotinamide mononucleotide

NAD = Nicotinamide adenine diphosphate

FIGURE 1 NAD metabolism in nucleated cells.

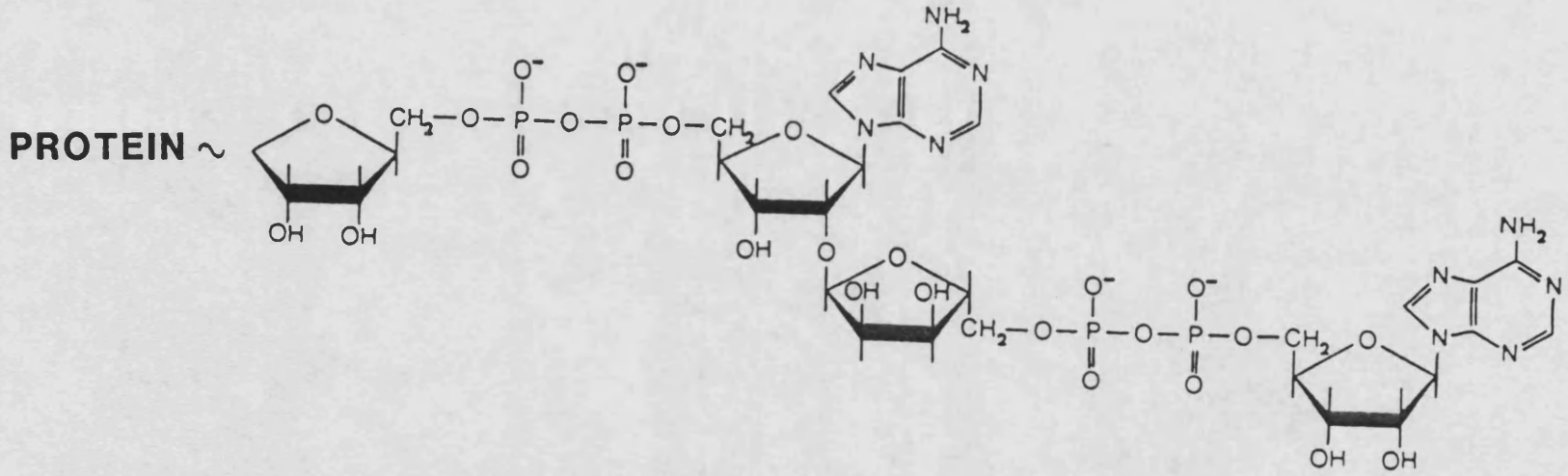


FIGURE 2 Structure of poly (ADP - ribose).

3) Elongation (addition of ADP-ribose) units on pre- existing mono - or oligo - (ADP-ribose)

The linear and branching forms of poly (ADP-ribose) have the same alpha (1-2) ribose-ribose linkage (Miwa *et al.*, 1981). An acceptor linked ADP-ribose unit was demonstrated to be elongated by terminal addition of new ADP-ribose units (Ueda *et al.*, 1979).

The precise role and mechanism of automodification on the surface of the enzyme is still not fully understood. Reports from two laboratories are contradictory to one another. Taniguchi (1987), using a pulse-chase experiment, first labelled ADPR transferase with [^{3}H] NAD (pulse-labelled) in the presence of Mg^{2+} and then chased with [^{14}C] NAD under the same conditions after complete washing of [^{3}H] NAD. Poly (ADP-ribose) was then digested with snake venom phosphodiesterase and analyzed. The [^{3}H]-labelled product was identified as iso-ADP-ribose and the only terminal AMP was detected as [^{14}C]-labelled AMP (see figure 2). Results indicate that the initially attached ADP-ribose unit at the automodification site was successively elongated by the addition of a new ADP-ribose unit to the terminal AMP only. However, results from Ikejima *et al.* (1987) using pulse-chase experiment, first with radioactive NAD then followed by non radioactive NAD, have shown that the new 'ADP-ribose' residues are added at 1" terminus, adjacent to ADPR transferase, such that the first ADP-ribose unit links to the next at the catalytic site. This is then extended out to become the terminal ADP-ribose. The same group also found that poly (ADP-ribose) glycohydrolase digests poly (ADP-ribose) with an endonucleotic incision and then removes the ADP-ribose residues progressively in the 2'→1" direction (ie toward ADPR transferase) (Ikejima and Gill, 1988). They have suggested that the two mechanisms which move in the opposite

direction will allow a single poly (ADP-ribose) chain to be extended at one end and simultaneously degraded at the other end, thus controlling the quantity and distribution of polymer around the DNA break.

The number of chains and the chain lengths of poly (ADP-ribose) can be estimated by isolation and quantification of the products of snake venom phosphodiesterase digestion. This was the first enzyme found to hydrolyze poly (ADP-ribose) (Nishizuka *et al.*, 1968) producing 5'-AMP from the terminus and a unique molecule, 2-(5"-phosphoribosyl)-5'-AMP (PR-AMP) (see figure 3) from each internal residue. These two products can be separated by thin layer chromatography (Nishizuka *et al.*, 1969) providing a quantitative estimate of the chain length. The product PR-AMP can be digested with alkaline phosphatase but it is resistant to digestion by snake venom phosphodiesterase. In contrast, ADP-ribose is digested by snake venom phosphodiesterase into ribose phosphate and 5'-AMP (Ikejima *et al.*, 1980; Wielckens *et al.*, 1981).

The products, 5'-AMP, PR-AMP and ADP-ribose can be quantitatively determined by radioimmunoassay using antisera highly specific for the respective product. Bredehorst *et al.* (1978) used alkali treatment to cleave mono ADP-ribose residues to 5'-AMP and ribose-5-phosphate and then used highly specific antibodies raised against 5'- AMP to quantify ADP-ribose by radioimmunoassay. Following the enzymatic degradation of the polymer, antibodies raised against 5'- AMP and PR-AMP were used to determine the polymeric ADP-ribose level. Adamietz and Bredehorst (1981) later used alkali in the presence of Mg^{2+} ions to degrade the ADP-ribose polymer directly to PR-AMP and 5'-AMP (instead of using degrading enzymes, which could give incomplete digestion) and suggested that such a cleavage method would allow determination of chain lengths *in vivo*. On the other hand,

ribosyl adenosine can be quantitatively estimated by HPLC. This is usually performed after the conversion of the compound to a fluorescent analogue (Juarez-Salinas *et al*; 1979; Sim *et al.*, 1980). The unique nucleotides ribosyl adenosine and diribosyl adenosine are released from linear and branched ADP - ribose polymers by digestion with snake venom phosphodiesterase and poly (ADP-ribose) glycohydrolase (Miwa and Sugimura, 1971; Sims *et al.*, 1980; Juarez-Salinas *et al.*, 1983). The fluorescent derivatives are then separated by HPLC and measured.

In animal tissues, poly (ADP-ribose) may be hydrolysed by two classes of enzymes. One is poly (ADP-ribose) glycohydrolase, an exoglycosylase, which cleaves the ribose-ribose bonds and also the branch region of poly (ADP-ribose) containing ribose-ribose-ribose to yield ADP-ribose. Poly (ADP-ribose) glycohydrolase has been purified from many tissues ie from calf thymus (Hatakeyama *et al.*, 1986), human erythrocytes (Tanuma *et al.*, 1986b) and from the nuclei of guinea pig liver (Tanuma *et al.*, 1986a). The second degrading enzyme is a phosphodiesterase which splits the phosphate-phosphate bonds in the polymer producing phospho-ribosyl-AMP and 5'-AMP (Futai *et al.*, 1968) (figure 3). Phosphodiesterase may be endonucleolytic or exonucleolytic (Miwa and Sugimura, 1982). The pH optimum for poly (ADP-ribose) glycohydrolase is pH 7.6 whereas the pH optimum for phosphodiesterase is about pH 10. Hence, poly (ADP-ribose) glycolhydrolase is the more physiologically important enzyme for degradation. Moreover, analysis of the degradation products of the polymer *in vivo* indicated that the glycohydrolase is mainly responsible for polymer degradation in various tissues (Miwa *et al.*, 1975).

1.1.3 NUCLEAR ADPR TRANSFERASE

ADPR transferase is a chromatin bound enzyme which is responsible for nuclear

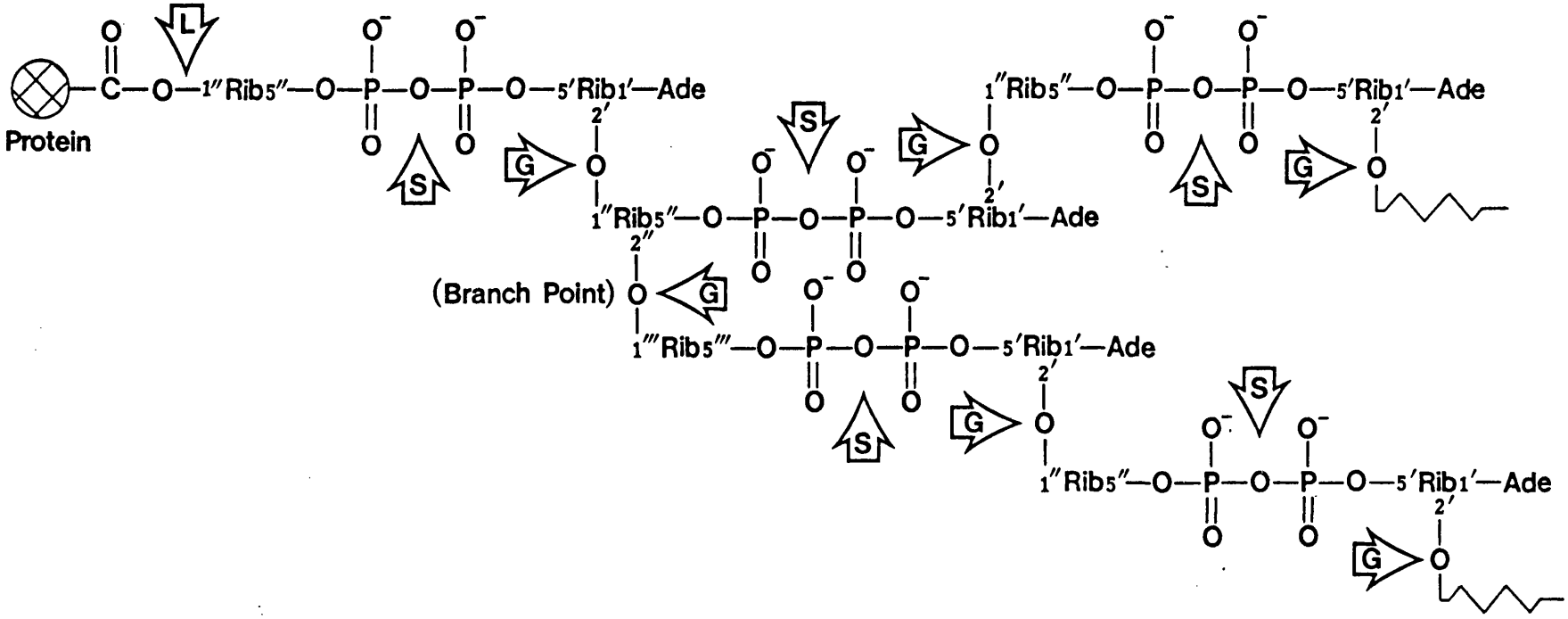


FIGURE 3 Schematic representation of poly (ADPR) metabolism.

The sites of cleavage by ADP-ribosylation protein lyase(L), snake venom phosphodiesterase (S), and poly ADPR glycohydrolase (G) are indicated by arrows. (White, 1988).

ADP-ribose polymerization. It transfers ADP-ribose residues to various protein acceptors with the release of nicotinamide and hydrogen ions. This enzyme is present in the nuclei of both animals and plants. It is also found in lower organisms such as the dinoflagellate *Crypthecodinium cohnii* (Werner *et al.*, 1984) and the slime mould *Physarum polycephalum* (Brightwell *et al.*, 1975). However, this enzyme activity is reported to be absent in many differentiated cells eg mature granulocytes (Ikai *et al.*, 1980a, b) epidermal cells (Ikai *et al.*, 1981) and in intestinal epithelial cells (Porteous and Pearson, 1982). The enzyme activity is localized in the nucleus of eukaryotic cells and 80% to 90% of it was shown to be associated with chromatin (Ueda *et al.*, 1968). Some cytoplasmic ADPR transferase has also been found in mitochondria of rat liver (Masmoudi and Mandel, 1987) and in ribosomes (Concha *et al.*, 1985). The latter case, the enzyme was found to be associated with free messenger ribonucleoprotein particles and it is able to modify several proteins from these particles (Thomassin *et al.*, 1985).

ADPR transferase requires Mg^{2+} and thiol reagents for maximal activity (Carter and Berger, 1981a). Furthermore it has an absolute requirement for DNA and it is greatly activated by DNA strand breaks (Hayaishi and Ueda, 1982). Using gel retardation and electron microscopy, it was shown that ADPR transferase forms loops with DNA and that in the absence of single or double stranded breaks it preferentially binds to superhelical DNA (Gradwohl *et al.*, 1987). The activity of the enzyme, *in vitro*, could be inhibited through the process of itself being phosphorylated by protein kinase C (Tanaka *et al.*, 1987).

An interesting property of ADPR transferase is that it is solely responsible for catalysing both the initiating event, when the first ADP-ribose residue is attached to the acceptor sites, and the further covalent attachment to this or other ADP-ribose

moieties (Kawaichi *et al.*, 1980) Jones and Skidmore (1984) have proposed that the enzyme may contain more than one catalytic site, such that different catalytic sites exist for attaching ADP-ribose initially to the acceptor and for subsequent elongation.

The presence of multiple ADPR transferase enzymes was initially shown by the differing reports of molecular mass of calf thymus ADPR transferase purified by different laboratories (Tsopanakis *et al.*, 1978, Jongstra-Bilen *et al.*, 1981). However, Jonstra-Bilen *et al.* (1981) and Holtlund *et al.* (1981) found no evidence of tissue or species specific differences in molecular mass of the enzyme from amongst tissues of rat liver, pancreas and bovine brain and kidney. Agemori *et al.* (1982) suggested that species or tissue specificity of ADPR transferase might exist, based on amino acid composition differences between mouse testicle and calf thymus. The molecular weight of the purified enzyme was shown to vary from 62 kDa in pig thymus (Tsopanakis *et al.*, 1978) to 130 KDa in calf thymus (Yoshihara *et al.*, 1978). The molecular mass of 62 KDa is reported to be an underestimate since it has been shown that it is subject to highly specific endogenous proteolytic processing (Holtlund *et al.*, 1983a). Holtlund *et al.* (1981) compared the molecular masses of ADPR transferase in HeLa cells and pig thymus and found them to be identical at 112 KDa, a view supported by Kawaichi's finding that the purified rat liver enzyme is 110 kDa (Kawaichi *et al.*, 1980). Results obtained from Kameshita *et al.* (1984) using limited chymotryptic digestion has shown that the enzyme can be cleaved into 3 distinct domains of 54, 46 and 22 KDa; respectively capable of binding NAD, DNA and of accepting ADP-ribose. Further study using plasmin to cleave the enzyme revealed that the DNA binding site is in the N-terminal peptide and that the C-terminal peptide contains the NAD binding site (Buki and Kun, 1988).

The isolation of partial or full length cDNA clones encoding the human enzymes has provided new information on the enzyme structure. Human fibroblast ADPR transferase cDNA contains an open reading frame for a 1014 amino acid polypeptide corresponding to a calculated molecular weight of 113 KDa. The amino-terminal DNA- binding domain is highly basic and contains two homologous sequences (amino acid positions 2-97 and 106-207). These conserved sequences contain two putative zinc-binding motifs (Uchida *et al.*, 1987, Kurosaki *et al.*, 1987) which are probably involved in the interaction with DNA. It was also found that there is a high content of lysine and glutamic acid residues in the automodification domain with same short repeats of Glu-Val-Lys in the central part of the molecule. Furthermore, it was shown that the N-terminal portion of the 46 KDa fragment specifically binds ^{65}Zn as well as nick-translated DNA in Southern-Western blot experiments (Mazen *et al.*, 1988).

When ADPR transferase activity is increased, it also modifies chromatin structure and affects the enzymatic activity of other enzymes. Various enzymes are inhibited following ADP-ribosylation such as topoisomerases I and II (Ferro and Olivera, 1984) Ca^{2+}, Mg^{2+} endonuclease (Yoshihara *et al.*, 1975) and DNA polymerase$\alpha$and β (Yoshihara *et al.*, 1985). In contrast to the above, DNA ligase activity was found to be stimulated in the presence of poly (ADP- ribose) (Ohashi *et al.*, 1983) or by ADP-ribosylation (Creissen and Shall, 1982).

1.1.4 PROTEIN ACCEPTORS OF ADP-RIBOSYLATION

A variety of proteins have been identified as acceptors of ADP- ribosylation. Among these, both histones and non-histone proteins have been identified. Histones H1 and H2B are the main acceptors of the monomeric or small oligomeric

modification (Kawaichi *et al.*, 1981a). ADPR transferase is a main acceptor of large polymers *in vitro* (Ogata *et al.*, 1980) and *in vivo* (Kreimeyer *et al.*, 1984). In the nuclei of the cellular slime mould *Physarum polycephalum* histone H1 is found to be a major acceptor protein (Poirier *et al.*, 1987).

Other proteins and enzymes have been found to be acceptor proteins, amongst these are the Ca^{2+}, Mg^{2+} dependent endonucleases (Tanaka *et al.*, 1984), DNA Topoisomerase I (Ferro and Olivera, 1984), RNA polymerase I (Taniguchi *et al.*, 1985), terminal deoxynucleotidyl transferase (Tanaka *et al.*, 1986) and seminal ribonuclease (Suzuki *et al.*, 1986). P^1, P^4 - BiS (5'-adenosyl) tetraphosphate (Ap4A), which is thought to be a positive growth signal and a trigger for DNA replication (Grummt, 1978), can be modified by ADP-ribosylation *in vitro* provided that Ap4A is attached to protein (Yoshihara and Tanaka, 1981). In a mouse hepatoma cell line (Hepa), an 80-KDa protein, (P80) was found to be an acceptor protein under conditions of essential amino acid starvation (Ledford and Jacobs, 1986).

Recently, Matsuura *et al.* (1988) have reported that *in vitro*, L- type pyruvate kinase purified from pig liver can serve as an acceptor for ADP-ribosylation by hen liver nuclei ADPR transferase and that this modification results in suppression of the subsequent phosphorylation by cAMP-dependent protein kinase.

Some of the links between ADP-ribose and its protein acceptors are via an ester bond between the C-1 hydroxy group of the distal ribose on an initial ADP-ribose moiety and the carboxy group of glutamic acid, or a terminal lysine residue (Burzio *et al.*, 1979; Adamietz and Hilz, 1976). These ester linkages are sensitive to hydrolysis in either neutral hydroxylamine or alkali. In contrast, some links are

hydroxylamine and/or alkali resistant (Kawaichi *et al.*, 1981b).

1.1.5 INHIBITORS OF ADPR TRANSFERASE

A major approach in the investigation of the involvement of ADP- ribosylation in cell structure and function has been to determine the physiological effects of ADPR transferase inhibitors. Thus they are widely used in defining a role for nuclear ADP- ribosylation. Early reports showed that NADH, α-NAD and NAD analogues significantly inhibited enzyme activity at concentrations equimolar to the substrate (Preiss *et al.*, 1971; Hilz *et al.*, 1974). Nicotinamide has been observed to be a competitive inhibitor of the enzyme (Fujimura *et al.*, 1967; Preiss *et al.*, 1971). Various analogues such as benzamide (Shall, 1975), pyrazimide (Khan, 1977) and picolinamide (Sim *et al.*, 1982) are also good inhibitors. Thymidine is a good inhibitor as is its close analogue 5-bromo-2 - deoxyuridine (Preiss *et al.*, 1971). Other compounds were also reported to be ADPR transferase inhibitors eg methylated xanthines, cytokinine (Levi *et al.*, 1978); theophylline, theobromine and caffeine (Haldorsson *et al.*, 1978). 3-Aminobenzamide, 3- methoxybenzamide (benzamide analogue) and 3-acetamidobenzamide (3- aminobenzamide analogue) (Purnell and Whish, 1980) are the latest ADPR transferase inhibitors introduced. These are widely used in many laboratories as tools to probe for the biological roles of nuclear ADP-ribosylation.

The intact carboxamide group of nicotinamide or picolinamide is essential for effective inhibition as shown by the dramatic disappearance of inhibition effect if the carboxamide group is modified to 3-acetyl or N-methyl carboxamide or N.N dimethyl carboxamide. More evidence that the intact carboxamide group on an aromatic parent ring is vital for inhibition was demonstrated in benzamide and

pyrazinamide. Purnell (1980) found that 3-aminobenzamide is more effective than benzamide, but 3-aminobenzoic acid is not. Substitution of the benzene ring with amino groups at position 2 or 4 decreases the inhibitory effect, 2-aminobenzamide and 4- aminobenzamide are less effective than 3-aminobenzamide and benzamide respectively. Substitution of a methyl group on the ring nitrogen as in 1-methylnicotinamide caused a large increase in ADPR transferase inhibition. Furthermore, it was also demonstrated that compounds in which the ring is completely saturated cause no inhibition eg nipecotamide and isonipecotamide (Sims *et al.*, 1982). These results indicate that a planar aromatic ring system is required for binding to the ADPR transferase. Another factor which may affect enzyme inhibition binding is the number and position of the methyl group on the xanthine ring as it was shown that the order of efficiency of methylxanthines was theophylline > theobromine > caffeine with ki values of 15.2, 29.8 and 244 respectively (Claycomb, 1976).

The majority of ADPR transferase inhibitors lack physiological specificity; 5-methylnicotinamide, thymidine and its analogues and the methylated xanthines affect many cellular processes other than ADP-ribosylation. For example, nicotinamide is a substrate for NMN pyrophosphorylase and may deplete the cellular phosphoribosylpyrophosphate pool (see figure 1) as suggested by Hilz's group (Liber *et al.*, 1973). Furthermore, nicotinamide, thymidine and methylated xanthines have all been shown to inhibit NAD glycohydrolase activity in rabbit reticulocytes (Webb, 1966; Wu *et al.*, 1978). Skidmore *et al.* (1979) reported similar results for 5-methylnicotinamide and theophylline in L1210 cells. Methylated xanthines also inhibit cAMP phosphodiesterase (Levi *et al.*, 1978). Grunfeld and Shigenaga (1984) found that nicotinamide and benzamide inhibit deoxyglucose uptake in differentiated 3T3-L1 fat cells. This finding was confirmed by Schechter

(1984) who reported that nicotinamide inhibits basal and insulin stimulated lipogenesis in rat fat cells and that most of the lipid found in adipocytes was synthesized from glucose (Kuri-Horcuch *et al.*, 1978). Thus any alteration of cellular processes observed on treatment of cells with the above compounds can not be ascribed directly to inhibition of ADPR transferase.

The specificity of 3-aminobenzamide, 3-methoxybenzamide and 3-acetamidobenzamide has also been challenged by various workers. The Cleaver group reported that 3-aminobenzamide caused a disturbance in purine metabolism (Cleaver *et al.*, 1983) and that it may affect other metabolic processes such as DNA synthesis, glucose metabolism and cell viability (Milam and Cleaver, 1984). Recent reports using the HPLC separation of the deoxynucleosides procedure has shown that 3-aminobenzamide exhibits a wide range of effects on DNA precursor metabolism, such as the enhancement effect of 3- aminobenzamide on [^{3}H] deoxycytidine incorporation into DNA relative to the other three deoxynucleotides. The incorporation of radioactivity from [^{3}H] glucose into the pyrimidine moiety of the bases in DNA was significantly inhibited by 3-aminobenzamide relative to incorporation into the purine fraction. A major inhibitory effect on the incorporation of the methyl group from [^{3}H] methionine into the deoxynucleotides reflects the metabolism of the one-carbon pool, thus involving the metabolic pathways of tetrahydrofolic acid, glucose and phospholipids (Milam *et al.*, 1986).

Results obtained from other groups however are contradictory to the above. Ben-Hur *et al.* (1985) studied the enhanced radiation lethality of chinese hamster cells using high concentrations of ADPR transferase inhibitors but only for short periods (2 h). Although results show some relatively small effect on the metabolism of glucose and on the synthesis of DNA, they suggested that these perturbations are

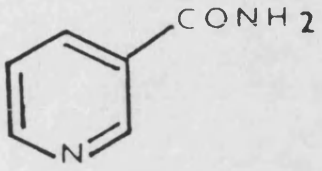
not correlated with an enhanced radiation response which, itself, was found to be due to the potency of the inhibition of poly (ADP-ribose) synthesis. Berger *et al.* (1987) have also demonstrated that the predominant effect of 3-aminobenzamide on mitogen stimulated human T lymphocytes is on ADP-ribosylation. Eventhough 3-aminobenzamide affected the rate of glucose utilization, the levels of ATP in cells were maintained close to those observed in cells treated with mitogens alone.

Discrepancies were demonstrated earlier by Hüting *et al.* (1985); their results showed that inhibition of ADPR transferase causes little or no growth inhibition and has no effect on purine or pyrimidine nucleotide synthesis *de novo*. The inhibition effects of ADPR transferase inhibitors on glycine incorporation into ATP and GTP and on thymidine incorporation into DNA were suggested to be related to an inhibition of ADP-ribose or that it may be a secondary effect of the inhibitors.

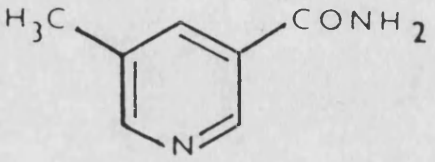
1.1.6 CELL CYCLE AND ADP-RIBOSYLATION

It was reported earlier that ADPR transferase activity was low in S phase and high in G_2 phase (Hilz and Stone, 1976; Purnall *et al.*, 1980). Recently, Leduc *et al.* (1988) have used an immunological and biochemical approach to study the properties of ADPR transferase during the cell cycle progession of G_1, S and G_2+M in rat FR 3T3 fibroblasts. This method is an alternative procedure to avoid possible artifacts due to classical methods of synchronizing cells which use drug treatment or deprivation of essential growth factors. Results confirmed that enzyme activity increased at the G_1/S boundary and reached a maximum in G_2 and M phase. These results suggest a cell-cycle activation of enzyme which occurs in the G_2+M phase. Moreover, it was shown that quiescent cells showed a lower content of ADPR transferase than cells in G_1 phase.

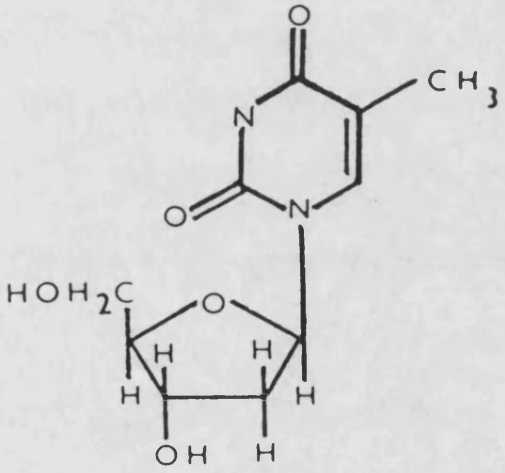
FIGURE 4.a Inhibitors of ADPR transferase.



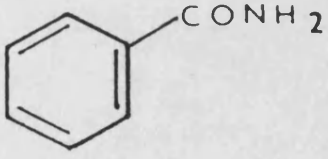
NICOTINAMIDE



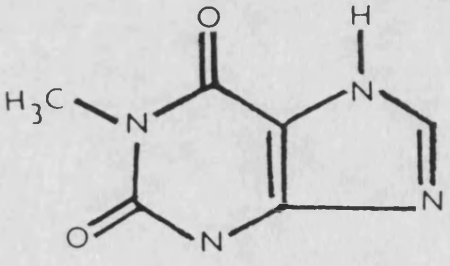
5-METHYL-NICOTINAMIDE



THYMIDINE

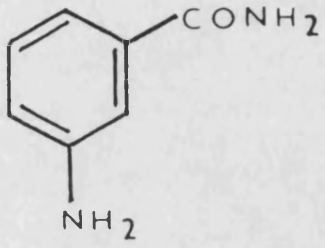


BENZAMIDE

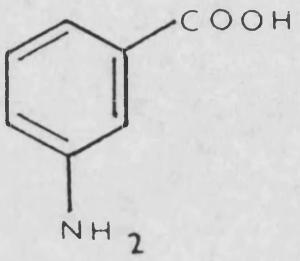


THEOPHYLLINE

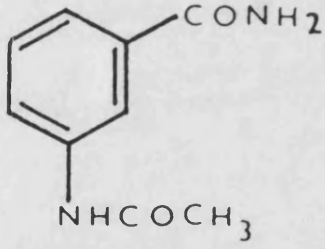
FIGURE 4.b Inhibitors of ADPR transferase and their acid analogues used in this thesis.



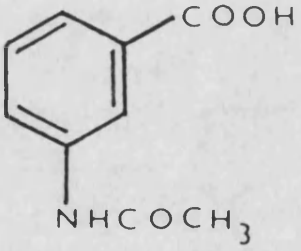
3-AMINOBENZAMIDE



3-AMINOBENZOIC ACID



3-ACETAMIDOBENZAMIDE



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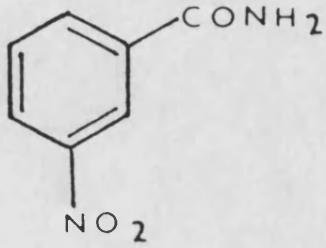
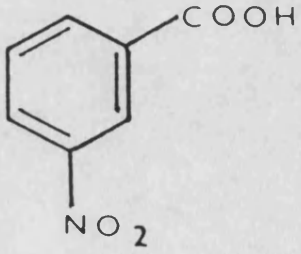


3-NITROBENZAMIDE



3-NITROBENZOIC ACID

Using [^{32}P] NAD incorporation in permeabilized cells, Holtlund *et al.* (1980) had shown that ADPR transferase exists in metaphase chromosomes. The same group reported that the increase in poly (ADP-ribose) occured mainly on histone Hl, not non-histone protein in metaphase and interphase arrested HeLa S3 cells (Holtlund *et al.*, 1983b). This phenomenon was similar to that shown in the results from Tanuma and Kanai (1982).

Adolph and Song (1985) investigated the basic differences in the ADP-ribosylation of non-histone protein from interphase nuclei and metaphase chromosomes in the HeLa cell genome. Results showed that the main acceptors of the [^{32}P] labelled NAD were from interphase. Further results from *in vivo* studies have shown that histones H2B, H2A and H4 were the predominant acceptors of the [^{3}H] adenosine label in interphase nuclei and that ADPR transferase was the only major acceptor for metaphase chromosomes (Adolph, 1987a). It was suggested that chromatin fibers of metaphase chromosomes are highly condensed in comparison to nuclei and the decrease in ADP- ribosylation at mitosis could be caused directly by the compaction of chromatin, making the proteins inaccessible to ADPR transferase. Furthermore, the higher degree of nicking DNA during interphase might stimulate the increased modification of nuclear proteins (Adolph, 1987b).

1.1.7 DNA METABOLISM AND GENE EXPRESSION

Chromatin structure plays an important role in the control of gene expression in eukaryotic cells (Eissenberg *et al.*, 1985) and ADP-ribosylation has been shown to be related to DNA metabolism and gene expression. Tanuma and Johnson (1983) reported that glucocorticoid treatment of mouse mammary 34I tumour cells decreases ADP-ribosylation of various nuclear proteins, especially of HMG14 and

17, concurrent with the induction of mouse mammary tumour virus gene expression. A decrease in ADP-ribosylation of HMG14 and 17 could cause subtle changes in chromatin structure making promotor regions more accessible to RNA polymerase II, the important enzyme in the regulation of eukaryotic gene expression (Manley *et al.*, 1980; Weil *et al.*, 1979). Furthermore, the same groups (Tanuma *et al.*, 1987) have demonstrated that 3-aminobenzamide, an inhibitor of ADPR transferase has the same capacity as dexamethasone (a synthetic glucocorticoid) to induce the synthesis of metallothionein and that the inductions of metallothionein were temporarily correlated with a decrease in the amount of endogenous poly (ADP-ribose) on non-histone HMG14 and 17.

The effects of steriod hormones on ADP-ribosylation are also of interest as these hormones are clearly established as being able to influence gene expression. It was reported that testosterone deficiency induced by castration was found to lead to a decrease in total ADP-ribose residues in mouse kidney and it was restorable upon daily injection with the hormone (Gartmann *et al.*, 1981). Oestrogen was found to stimulate ADPR transferase in quail oviduct (Muller *et al.*, 1974) and progesterone was found to induce ADPR transferase activity in *Xenopus oocytes* (Burzio and Koide, 1977).

Aubin *et al.* (1983) and Poirier *et al.* (1982) have shown that *in vitro* ADP-ribosylation caused the relaxation of the polynucleosome structure, presumably through its effect on modification on histone H1. In addition to this discovery, *in vivo* studies have shown that the activity of topoisomerse I, an enzyme implicated in DNA replication, transcription and modification of chromatin structure (Gellert, 1981) was decreased in parallel to the extent of its ADP-ribosylation (Jongstra-Bilen *et al.*, 1983). This evidence has suggested that the involvement of ADP-ribosylation

is related to DNA metabolism and gene expression *in vivo* through alterations of the chromatin structure.

Further evidence was shown by Sastry and Kun (1988) who suggested that binding of poly (ADP-ribose) to DNA and nucleosome could have a localized effect on the topology of DNA which may indirectly influence gene expression. Recently it has been reported that a significant portion of endogeneous poly (ADP-ribose) is tightly associated with the nuclear matrix (Cardenas-Corona *et al.*, 1987), a major ADP-ribosylation site within the nucleus, in which the metabolic reaction may be closely connected with the events modulating DNA replication (Alvarez-Gonzalez and Ringer, 1988). It was found that nuclear matrix contains an active fraction of DNA replication enzyme complexes (Tubo and Berezney, 1987).

1.1.8 CELLULAR DIFFERENTIATION

Caplan and Rosenberg (1975) were the first to demonstrate that ADPR transferase was involved in cellular differentiation using mesodermal cells of embryonic chick limb buds, since these cells have the ability to differentiate into muscle or cartilage. The authors showed that the NAD levels fluctuated during differentiation. Later Yong and Sweeny (1978) showed a decreased incorporation of adenosine in mouse ova during and after fertilization.

The precise mechanism for the involvement of ADP-ribosylation in cell differentiation is still unclear. It is not certain whether DNA strand breaks or chromatin structural changes resulting in an increased availability of ADP-ribose, are responsible for these changes in enzyme activity. Some drastic changes in ADP-ribosylation which may affect cytodifferentiation could not be ascribed to DNA

strand break and the signal prompting these changes has not been identified (Brac *et al.*, 1985). It is possible that these alterations in the level of ADP-ribosylation reflect the varying availability of NAD during cytodifferentiation.

Farzaneh *et al.* (1982) and Johnstone and Williams (1982) suggested that spontaneous DNA strand breaks are responsible for activating the ADPR transferase activity during early differentiation events in chick myeloblasts and human lymphocytes. Farzaneh *et al.* (1987a) have reported that during the induced differentiation of the human promyelocytic leukemic cell line, HL 60, strand-breaks are formed and transiently maintained along the myelocytic lineage DNA before being religated and that such processes require ADPR transferase. The same group has also demonstrated that the efficiency of DNA repair is the same both in the differentiated and undifferentiated HL 60 cells (Farzaneh et *et al.*, 1987b). Results from Hacham and Ben-Ishai (1984) and Althaus *et al.* (1982b) are contradictory to the above findings. They reported that transient and spontaneous alterations in ADPR transferase activity in primary hepatocyte cultures are not causally related to DNA fragmentation. Furthermore, Jackowski and Kun (1981) found age-dependent variations in ADPR transferase activity in cardiocyte nuclei in the absence of measurable DNA fragmentation.

More evidence was based on ADPR transferase inhibitors. They were found to prevent the induction of adipocytes (Lewis *et al.*, 1982) and Friend cell differentiation (Terada *et al.*, 1979). In *Drosophila melanogaster*, ADPR transferase inhibitors have been shown to retard development, kill larvae in a dose-dependent manner and induce mitotic recombination in response to γ irradiation (Ferro *et al.*, 1984). Suzuki *et al.* (1987) have shown that RNA expression of ADPR transferase decreased during the granulocytic differentiation of HL 60 cells upon induction by

retinoic acid. Furthermore, the levels of the enzyme molecule and mRNA for the ADPR transferase were reduced by 50% of the original during nerve growth factor-promoted neurite outgrowth in rat pheochromocytoma PC12 cells. These results suggested that the decrease of ADPR transferase activity may be due to a reduction in gene expression. This result suggests that such reduction seems to be required for some cellular differentiation (Taniguchi *et al.*, 1988).

1.1.9 DNA REPAIR

Roitt (1956) was the first to show that treatment of cells with alkylating agent induces cellular NAD depletion and subsequently prevents glycolysis. Later, Sims*et al.* (1983) demonstrated that NAD depletion is followed by a dramatic decrease in ATP levels causing a reduction in ATP-dependent functions leading eventually to cell death. Whish *et al.* (1975) and Smulson *et al.* (1975) have reported independently that DNA damaging agents stimulated the activity of ADPR transferase followed by a decrease in cellular NAD level. These observations provided a rational explanation for the drop in cellular NAD and a suggestion that poly (ADP-ribose) may be involved in the cytotoxic effects of alkylating agents.

The dependency of poly (ADP-ribose) synthesis on DNA strand breakage has been extensively reported (Berger *et al.*, 1979; Benjamin and Gill, 1980a and Jacobson *et al.*, 1983). The type of break introduced into the DNA duplex is important in regulating ADPR transferase activity. Double-stranded restriction fragments with flash ends are reported by Benjamin and Gill (1980b) as being most effective, three times more so than DNA fragments with 3' extensives which are more than three times as effective as DNA with unpaired nucleotides extending from the 5' termini or DNA with single strand breaks.

A role for poly (ADP-ribose) in DNA repair has been suggested by Durkacz *et al.* (1980a, b) who found that 3-aminobenzamide or 5- methylnicotinamide retarded the rejoining of DNA strand breaks induced by dimethyl sulphate (DMS) in mouse L1210 cells. Furthermore, ADPR transferase inhibitors were also found to inhibit the decrease in cellular NAD levels normally seen following DMS treatment in the absence of inhibitors. The same group also showed that ionizing radiation has little or no effect on the NAD level in these cells (Nduka *et al.*, 1980). These results were supported by James and Lehmann (1982). However, Zwelling *et al.* (1982) reported that both 3-aminobenzamide and 5-methylnicotinamide slowed but did not prevent the rejoining of x-ray induced DNA strand breaks in L1210 cells. These results suggested that x-ray break rejoining may happen in two ways, one associated with poly (ADP-ribose) synthesis and the other independent of its synthesis. James and Lehmann (1982) also suggested that the effectiveness of different agents at inducing poly (ADP-ribose) synthesis may be related to the level of DNA breaks induced. At an equitoxic dose, an alkylating agent will produce five times as many breaks as ionizing radiation and ten times as many as UV radiation (Shall, 1984).

Some reports however, claimed that ADPR transferase inhibitors stimulated strand rejoining after exposure to alkylating agents or UV light in human lymphoid cell types (Bohr and Klenow, 1981; Althaus *et al.*, 1982a).This authors suggested that the differences may be that the influence of such inhibitors on DNA repair is dependent on the agent used initially to stimulate the repair synthesis together with possible differences in cell membrane permeability to the inhibitors. Furthermore, there may be substantial differences in the kinetic parameters of the several steps in DNA repair which contribute to the observed differences in responsiveness to ADPR transferase inhibitors in various cell lines. These observations were supported by Durrant and Boyle (1982) who found in rodent cell lines that the enhancement of

cytotoxicity by ADPR transferase inhibitor is cell line dependent.

It is now accepted that ADP-ribosylation is involved in DNA repair though its exact role remains unclear. It was first postulated that the ADP-ribosylation is necessary for full activity of the DNA ligase step that is, the final step, of DNA repair (Shall, 1984). This was concluded from data showing a higher steady state concentration of DNA strand breaks in alkylated cells when ADPR transferase inhibitors were present (Ohashi *et al.*, 1983). Criessen and Shall (1982) have suggested that DNA ligase II is involved in the process as they found, *in vitro*, the direct modification of DNA ligase II. However, the *in vivo* experiments have failed to demonstrate a modification of the DNA ligase by poly (ADP-ribose) (Kreimeyer *et al.*, 1984; Adamietz and Rudolph, 1984). Some reports suggest that such higher steady state concentrations of DNA strand breaks in alkylated and ADPR transferase inhibitors compared to alkylated cells alone do not necessarily result from a slow-down of the repair rate. It was suggested that it could be the consequences of a higher number of incisions either by repair endonucleases or by endonucleases not directly involved in DNA repair eg Ca^{2+}/Mg^{2+} dependent endonuclease, which increased DNA breaks by further cutting (Wielckens *et al.*, 1985).

Cleaver and Morgan (1985) have suggested that poly (ADP-ribose) does not regulate the ligation stage of repair. Their results have shown that the direct measurement of the rate of ligation of intracellular repair patches and of the size of repair patches are unchanged when ADP-ribosylation is inhibited. They argued that if the process of ligation is delayed by inhibition of poly (ADP- ribose) synthesis, continued repair polymerization should therefore generate longer repair patches. This notion was supported by Collins (1987) who blocked repair synthesis in UV treated hamster cells with inhibitors of repair synthesis. They reversed the inhibition

processs and investigated the ligation process of excision repair using an ADPR transferase inhibitor. Results showed that 3-aminobenzamide does not delay the rejoining of the DNA break. Furthermore, some reports have even suggested that 3-aminobenzamide actually stimulated ligation of repair patches (Cleaver and Park, 1986). If used at very low concentrations (0.01-0.1 mM) it was found to reduce strand-break frequencies and increase repair replication in human lymphoid cells damaged by MMS (Cleave and Morgan, 1987).

In contrast to the above, Hunting and Gowans (1987) reported that 3-aminobenzamide inhibited ligation of repair patches in normal human fibroblasts following UV irradiation. Their experiment was different from Collins' (1987) in that it was not carried out in the presence of inhibitors of repair synthesis as they were shown to reduce NAD concentration. However, UV radiation alone did not lower NAD concentration. This finding led to the suggestion that the results obtained from Collins (1987) and Cleaver and Park (1986) which show high levels of strand breaks while using inhibitors of repair synthesis to determine the effect of 3-aminobenzamide on repair, may give misleading results since poly (ADP-ribose) synthesis may already have been inhibited by the depletion of NAD.

The studies on chromatin structure in cells cultivated in the presence of benzamide have indicated that ADP-ribosylation is involved in the organisation of chromatin structure, which allows correct repair of DNA following alkylation (Wielckens *et al.*, 1985). One example is the relaxation of chromatin caused by the ADP-ribosylation of histone H1 (Poirier *et al.*, 1982; Aubin *et al.*, 1983). This could permit excision of DNA lesions by making the relax regions more accessible to the repair enzyme. Reports obtained from Neidergang *et al.* (1985) have also suggested a potential role for poly (ADP-ribose) in DNA replication and repair as it was shown

that decondensed ADP-ribosylated polynucleosomes were a better template for DNA polymerase. De Murcia *et al.* (1985, 1986) observed that under poly (ADP-ribose) turnover conditions, local chromatin remained in a decondensed form. Creation of these open chromatin regions may be the key modification in which poly (ADP-ribose) is necessary for the DNA repair processes as postulated by Mathis and Althaus (1987).

Recently, Loetscher *et al.* (1987) reported that the level of post-translation by poly (ADP-ribose) modifications of chromatin proteins in mammalian cells is related to the availability of NAD which varies in different physiological and pathological states. Loetscher suggested that poly (ADP-ribose) may serve as a signal which alters metabolic conditions involved in chromatin and thus modulates its functions in accordance with a change in metabolic state.

PART II

1.2.1 TYPES OF GENETIC CHANGES

Gene mutation is a heritable change in the nucleotide sequence of DNA or of its genoming RNA (in the case of RNA viruses). This change may be expressed, for example, as a change in structure of a protein which alters or abolishes its enzyme properties. Changes in the content or arrangement of information in DNA can occur in a single nucleotide within a codon or in the number of complete chromosomes in a genome. There are three general types of mutation; point, chromosomal and genomic mutation. (Venitt and Parry, 1984)

Point mutations are changes in nucleotide sequence in one or few codons and these can occur by base substitution, deletion or addition. Additions or deletions can change the reading frame of DNA and when this occurs, they are known as frameshift mutations. The change of codon may lead to the insertion of the wrong amino acid into the polypeptide. If this occurs at a critical site in a polypeptide, this may inhibit its function eventually leading to partial cell growth, or it may cause an increase in temperature sensitivity rather than a total loss in function. The change may also lead to the conversion of a codon normally specifying an amino acid to one which is a terminator codon. This causes polypeptide synthesis to stop prematurely with the production of defective proteins containing short polypeptide fragments. Point mutations are usually detectable by the effects of the expression in the mutant cell or individual as a change in phenotype.

Chromosomal mutations are recognised as morphological alterations in the gross structure of chromosomes. They occur during breakage and reunion of

chromosomal material during the cell cycle. It may happen as inversions (a length of chromosome is inserted back-to-front) or translocations (one section of chromosome becomes attached to another at the wrong place). This type of mutation normally has dramatic consequences for gene expression, most of which are lethal to the individual.

Genomic mutations are the changes in the number of chromosomes in the genome. The normal diploid (Wild-type) contains a complete set of chromosomes from each parent. Polyploidy occurs where the diploid genome is doubled or tripled. Loss or gain of a single chromosome is known as aneuploidy, the addition of one chromosome is trisomy and deletion of one chromosome is monosomy.

From the time that mutation was emphasized by de Vries (1901) as a fundamental genetic process, its analysis has been one of the most important areas in research. It was recognized as the cause of modified genes which constitute the raw material for evolution and which are subject to natural selection. This is such a crucial process that modern definitions of life include mutability as a fundamental property of living organisms.

Mutations may occur spontaneously or may be induced by physical and chemical agents (Singer and Kusmierek, 1982). The first of several major advances in the analysis of mutational process was discovered by Muller (1927) who demonstrated that mutation could be induced in *Drosophila* using x-ray irradiation. This was eventually broadened to include mutagenesis by chemical agents (Auerbach and Robson, 1947). If the frequency of spontaneous mutations is too low for convenient experimentation, mutagens can be used to increase the frequency of mutants in culture at 10 to 1000 times the spontaneous frequency. Mutagens increase the

frequency of impaired bases escaping repair and thus leads to the change in the original sequence of DNA (Roberts, 1978; Lawley, 1974).

Although cell culture and mutant isolation procedures are slow due to the length of time required to obtain results, the progress of somatic cell genetics has developed into a recognized discipline. This is largely because of the development of methods for isolation of a variety of mutant phenotypes and the technology for manipulating the mutant gene in order to study the consequences of the changes.

1.2.2 REPLICA PLATING AND CLONING

Replica plating is useful for isolating mutants which can not be isolated by direct selection, for example, in cells expressing a conditional lethal phenotype such as temperature sensitivity or cells expressing or failing to express specific antigens. The technique screens large numbers of individual clones of cells for the phenotypes of interest while still maintaining a master copy of the colonies. This involves plating 100-400 cells per dish and applying one of a variety of materials eg filter paper, nylon or polyester cloth on top of the colonies so that some but not all of the cells from each of the colonies are transfered to the material from the dish. The replica can be screened for the desired phenotype while the master is maintained. When the copy of interest is identified by a particular assay, then one can return to the master dish and isolate cells from that colony. In this way, any test can be used to screen a population without requiring a selection by cell killing for the desired phenotype (Gal, 1987).

Methods for isolating single cells and propagating them into a population of cells are called cloning and the majority of them fall into one of the three categories of

techniques

1) Diluting techniques. This can be done using microtest plates containing 96 wells, each well holding up to 0.4 ml. A single cell can be added (diluted with sufficient volume of medium to give 10 cells/ml) in 0.1 ml of medium per well and thereby provide the individual cell with its own culture dish and with a sufficiently small amount of fluid to minimize the conditioning factors (Robb, 1970; 1973).

2) Cloning of Anchorage - Dependent cells. The cells are seeded onto 6 cm tissue culture dishes or petri dishes coated with either collagen or fibrin (Reid and Rojkind, 1979). By seeding only 100-200 cells per plate, large colonies of cells can be obtained that are sufficiently separated to permit the isolation of individual colonies (Schmitt and Maroudas, 1974).

3) Cloning of Anchorage - Independent cells. Dilute concentrations of cells are seeded into medium solidified by agar or agarose. Cells capable of growing in suspension in the agar medium form colonies (Puck *et al.*, 1956).

1.2.3 ISOLATION OF MUTANTS AND THEIR RELATED EFFECTS

In order to broaden the knowledge of the genetics and physiology of mammalian cells, a large variety of cells carrying defined genetic markers must be available. The selective procedures that have been worked out are those for obtaining cell lines with a particular nutritional deficiency (auxotrophic mutants), with conditional lethality (temperature- sensitive) and with resistance to any of a variety of drugs (drug-resistant).

Auxotrophic mutations are well suited to mapping synthetic pathways. Drug-resistance markers provide a means to study the properties of a target molecule in terms of its role in normal cell function. Conditional lethal mutations are useful for the study of the regulation. When the lethality is temperature-sensitive (ts), there is a potential for correlating the effects of temperature shift on cellular properties with changes in the functional or structural integrity of an isolated molecular component. Mutants therefore provide a means of evaluating the biological importance of enzymes or other proteins which can be isolated and characterized *in vitro*.

Auxotrophic mutants and conditional (mainly temperature- sensitive) mutants must be isolated either by nonselective procedures such as replica plating or by lethal growth method. This involves the creation of a situation wherein the normal cells grow and are killed leaving behind the mutants, which can be rescued.

The selective agents that have been used to isolate auxotrophic and temperature-sensitive mutants, are ones that act during the S phase of the cycle. These are "DNA poisons" such as cytosine arabinoside (Thompson *et al.*, 1970), 5 fluoro-2-deoxyuridine (FUdR) (Meiss and Basilico, 1972), the high specific activity of tritium [^{3}H]-thymidine (Thompson *et al.*, 1970) or 5-bromo-2-deoxyuridine (BUdR) and light (Naha, 1969).

Procedures for succesfully isolating auxotrophic mutants using BUdR and light were first developed by Puck and Kao (1967). In this procedure, a population of cells, most of which display wild-type growth characteristics with a few rare cells displaying additional mutant nutritional requirements, is placed in a selective medium. In this situation only the former but not the latter can grow. An agent,

such as BUdR, that will kill growing cells is then added to isolate different mutants (Kao and Puck, 1968; Puck and Kao, 1967). This procedure depends on the differential incorporation of the thymidine analogue (BUdR) into the DNA of dividing but not into non-dividing cells in a selective medium sufficient for growth of wild-type but not mutant cells. After incorporation of BUdR, the cell culture is exposed to visible light and as BUdR is light sensitive, the wild-type (dividing) cells are killed, leaving the mutant (non-dividing) cells. For details of this procedure see Patterson and Waldren (1987). These selection procedures have allowed for the isolation of a wide variety of mutants with new nutritional requirements (Patterson, 1985) or of mutants with altered temperature sensitivity (Patterson *et al.*, 1976). Many of auxotrophic mutants have been analyzed biochemically and they have been shown to involve a single gene mutation resulting in absence or malfunction of a specific enzyme. Many different auxotrophic mutants defective at steps in the synthesis of purines and pyrimidine, cholesterol, unsaturated fatty acids, amino acids and carbohydrate metabolism have been isolated (see table 1). The rate of spontaneous mutation frequency for auxotrophic mutants is in the order of about $x10^{-6}$ (Kao and Puck, 1974).

For temperature-sensitive mutants, the conditions of killing wild-type at the nonpermissive temperature were used by incubating cells with "DNA poisons". This takes into account that under such conditions, wild-type cells multiply normally while the mutants are arrested in some essential steps of their metabolism. Preferential killing of wild-type cells enriches the population with mutants. The cells then shifted back to the permissive temperature to allow for growth of mutant cells. Mutants that are temperature-sensitive for growth and that are blocked at specific points in the cell cycle enable specific control events in the cell cycle to be identified, for example, a chinese hamster mutant blocked at G_1 at 40 deg.C was

TABLE I Auxotrophic mutants (Puck and Kao, 1982)

Mutant	Nutritional requirement	Enzyme defect	References
pro⁻	Proline or pyrroline-5-carboxylic acid	Defective in converting glutamic acid to glutamic -semialdehyde	Ham (1963a; Kao and Puck (1967; 1974)
gly⁻	Glycine	Serine hydroxy-methytransferase	Chasin *et al.* (1974); Law and Kao (1979)
ade⁻	Adenine, hypoxanthine, their ribonucleosides, or ribonucleotides, or 5-aminoimidazole-4-carboxamide	Amidophosphori-bosyltransferase	Kao and Puck (1972)
ade⁻	"	Phosphoribosyl-formylglycinami-dine synthetase	Kao (1980) Patterson (1975)
ade⁻	"	Phosphoribosyl-glycinamide synthetase	Patterson *et al.* (1974)

TABLE 1 (continue)

ade⁻	"	Phosphoribosylamino imidazole carboxylase	"
ade⁻	"	Phosphoribosyl-glycinamide formytransferase	"
Urd⁻	Uridine, orotic acid or dihydroorotic acid	Carbamyl phosphate synthetase, aspartate trans-carbamylase, and dihydroorotase	Davidson *et al.* (1979) Patterson and Carnright (1977)
Urd⁻	Slower growth, not requiring uridine	Dihydroorotate dehydrogenase	Stamato and Patterson (1979)
Urd⁻	Uridine	Orotate phosphori-bosyltransferase and OMP carboxylase	Patterson (1980)
trans⁻	Unable to synthesize valine, leucine or isoleucine from its respective a-keto acid	Branched-chain amino acid transaminase	Jones and Moore (1976)
ala⁻	Alanine	Alanyl-tRNA synthetase	Hankinson (1976)

TABLE 1 (continue)

glu$^-$	Glutamate		"
ino$^-$	Inositol		Kao and Puck (1975)
GAT$^-$	Glycine-adenine-thymidine	Folylpolyglutamate synthetase	Jones *et al.* (1980)
pur$^-$	Like ade	Phosphoribosylformylgycinamide synthetase	Taylor *et al.* (1971)
Mutant #49	Unsaturated fatty acid	Microsomal stearoly-CoA desaturase	Chang and Vagelos (1976)
Mutant #215	Cholesterol	Defective in lanosterol demethylation	Chang *et al.*, (1977)
ser$^-$	Serine		Jones and Puck (1973)
AUXB1/ GAT$^-$	Glycine-adenosine-thymidine	Folylpolyglutamate synthetase	Taylor and Hanna (1977)
AUXB3/	Glycine-adenosine	Defective in folate	McBurney and Whitmore

TABLE 1 (continue)

GA$^-$		metabolism; not complementing AUXB1/GAT	(1974)
TSH1/leu$^-$	Auxotrophic for leucine at 39.5 deg.C, prototrophic at 34 deg.C	Defective in leucyl-tRNA synthetase at 39.5 deg. C	Faber and Deutscher (1976) Thompson *et al.* (1975)
PSV3/asn	Auxotrophic for asparagine at 39.5 deg.C, prototrophic at 34 deg.C	Defective in aspara gyl-tRNA synthetase at 39.5 deg.C	"
arg$^-$	Inability to utilize citruline in place of arginine	Argininosuccinate synthetase or agininosuccinase or both	Naylor *et al.* (1976)
cys	Inability to utilize cystathionine in place of cystine	Cystathionase	"
polyamine$^-$	Polyamine		Pohjanpelto *et al.* (1981)
Inosine$^-$	Unable to grow on inosine as the only	Purine nucleoside phosphorylase	Hoffee (1979)

TABLE 1 (continue)

	carbon source		
TdR^-	Thymidine, deoxycytidine or deoxyuridine	Inability to reduce UDP to dUDP	Meuth *et al.* (1979)
AT^-	Adenine-thymidine	Defective in tetrahydrofolate metabolism; complementing CHO-K1/pro GAT	Kao and Puck (1972)
Glucosa-mine	D-glucosamine or D-galactosamine		Onada and Matsushiro (1973)
asn/ts	Auxotrophic for asparagine at 39 deg.C, prototrophic at 35 deg.C	Defective in asparagly-tRNA synthetase at 39 deg.C	Wasmuth and Caskey (1976)
pur-1	Like ade	Amidophosphori-bosyltransferase	Feldman and Taylor (1975)
gln^-	Glutamine		Chu *et al.* (1969)
ght	Glycine-	Folylpolygluta-	Chu *et al.* (1972)

TABLE 1 (continue)

	hypoxanthine-thymidine	mate synthetase	
urd$^-$	Uridine		"
gal$^-$	Glucose; inability to utilize galactose		Sun *et al.* (1974)
B2/Res$^-$	Respiration-deficient, auxotrophic for CO and asparagine, requiring high glucose supply for glycolysis	NADH-Coenzyme	Scheffer (1974)
B9/Res$^-$	"	Succinate dehydrogenase	Soderberg *et al.* (1977)
asn	Asparagine	Asparagine synthetase	Goldfarb *et al.* (1977)
arg$^-$	Arginine requirement can be satisfied by argininosuccinate but not by citrulline	Argininosuccinate synthetase	Carritt *et al.* (1977)

found to be defective in glycoprotein synthesis (Tenner *et al.*, 1977). The defect of temperature-sensitive mutants is usually attributable to a single amino acid substitution in a protein essential to this process. As a result of such mutations, the protein remains functional at low temperature, but it loses its biological activity at a higher temperature which does not affect the wild-type gene product. Growth is then normal at low temperature, because the affected protein is stable under these conditions but at higher temperatures no growth is observed. (Siminovitch, 1981; Basilico, 1977) The rate of spontaneous mutation frequency for temperature sensitive mutants is in the order of 10^{-6}-10^{-7} (Basilico and Meiss, 1974).

The use of radioactively-labelled precursors provides a powerful method for the selection of mutants defective in membrane transport and in metabolic pathways. This method, called radiosuicide, is highly selective as the substrate itself is the killing agent in wild-type cells. The principle is based on the killing effects generated by the incorporation of radiolabelled precursor. Therefore, any mutant able to reduce the uptake and/or the metabolism of the radioactive toxic precursor will survive. The wide variety of cell lines derived from the application of this method demonstrates its usefulness as seen on table 2.

1.2.4 DRUG RESISTANT MUTANTS AND THEIR RELATIVE EFFECTS

The isolation of somatic cell mutants resistant to specific drugs with known specificity has proved to be the simplest way to produce specific mutations in genes which encode proteins of special interest. There are many advantages to the isolation of drug- resistant mutants as the drug targets the mutation to a specific protein or metabolic process. This allows initial biochemical characterization of the mutant to be much easier. Furthermore, it also permits the investigator to begin the

TABLE 2 Mutants isolated by radioactive- labeled precursors

Mutant Phenotype	Screening	References
The glucose transport and Glycolytic defective	2 Deoxy (^{3}H) glucose	Pouyssegur *et al.* (1980a)
Glucose oxidative pathway defective	(^{3}H) Glucose	Franchi *et al.* (1981) Pouyssegur *et al.* (1980b)
Transport of neutral amino acids defective	1)α-(methyl- (^{3}H) Aminoiso-butyric acid	Finkelstein *et al.* (1977)
	2) (^{3}H) Proline	Dantzig *et al.* (1982)
Unable to synthesize phosphatidyl choline	(^{14}C) Choline	Kuge *et al.* (1985)
Receptor-mediated endocytosis defective	(^{35}S) Mannose 6-phosphate proteins	Robbins *et al.* (1983)
Protein secretion defective	(^{35}S) Secreted bound to nitrocellulose membrane overlay	Nakano *et al.* (1985) Nakano and Akamatsu (1985)

genetic analysis of a system which would otherwise be complex.

Inhibitors that kill cells or arrest their growth can be used to select resistant mutants. Although several different mechanisms can mediate resistance, substantial evaluation of the biological activity or quantity of a protein mediating resistance is often but not always the direct target of the inhibitor.

One of the most frequent problems encountered when isolating drug-resistant mutants is the high frequency of appearance of multidrug resistance. Multidrug resistant cell lines are typically resistant to the anticancer drugs vinblastive, vincristine, actinomycin D and adriamycin, regardless of which of these was used as a selective agent. When tested, they are also frequently resistant to a range of other structurally and funtionally varied substances such as colchicine, pyromycin and emetine (Ling *et al.*, 1983; Gerlach *et al.*, 1986).

The most direct selection of drug-resistant mutants is a single-step procedure whereby a large culture of wild-type cells are exposed to a cytotoxic drug. The survival clones of drug-resistant phenotypes are then isolated. (This method is used in this thesis and employs an inhibitor of ADPR transferase as a cytotoxic drug). This procedure simplifies the characterization of the mutant since only one biochemical target should be involved in generation of phenotype. In order to isolate single-step mutants, it is necessary to choose the minimum concentration of a drug which will kill the great majority of cells and leave only the genetically resistant population. To obtain cell lines resistant to a high concentrations of a drug, it may be possible to extend the single-step isolation procedure to a multistep or a continuous selection. Successive increments in drug resistance might be obtained simply by submitting the cell population to consecutive single step

selections with gradual increases in drug concentration or maintaining a mass population under continuous selection pressure. They are then maintained in a gradually increasing inhibitor (drug) concentration, until cells with a satisfactory degree of resistance overgrow the culture or are frequent enough to be isolated at a low cell density. A disadvantage of this procedure is the difficulty of evaluating the number of mutant alleles or loci that might be necessary to produce the selected phenotype, since the frequency of mutant phenotypes in the wild-type population can not be measured directly.

The range of genetic mechanisms such as point mutation, deletion, duplication, chromosome instability and the generation of additional DNA copies by gene amplification are involved in development of inhibitor resistance. The simplest form of genetic change that may be considered is a base substitution in a gene leading to an amino acid change in a protein involved in resistance. Some forms of change such as gene amplification may be reversible in the absence of any continuous selective pressure.

Many drug resistance mutants are well known and have been studied in great detail. A number of investigators have already isolated spontaneous mutant clones resistant to 8-azaguanine (or related purine analogues) by using single-step selection procedures (Littlefield, 1963; Chu and Malling, 1968; Gillin *et al.*, 1972). They have shown that resistance commonly arises from the loss of activity of the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (Kit *et al.*, 1963; McKusick and Ruddle, 1977; Chinault and Casey, 1984). An enzyme, adenine phosphoribosyltransferase (APRT), the homologue of HGPRT, has also been studied. This enzyme is considered to be primarily responsible for the recycling of purine bases into nucleotide pools. The adenine analogues such as diaminopurine,

fluoroadenine have been used to select resistant lines which have lost APRT (Jones and Sargent, 1974; Chasin, 1974; Steglich and DeMars, 1982). Similarly, the pyrimidine analogue 5-bromodeoxyuridine (BrdU) has been used to select resistant lines which have lost thymidine kinase activity (Littlefield, 1965; Roufa *et al.*, 1973). A wide variety of other drugs have been used to select resistant phenotypes of somatic cells in culture (see table 3).

The simplest form of genetic change that may be considered is a base substitution in a gene, leading to an amino acid change in a protein involved in resistance. Simonson and Levison (1983) have demonstrated this by cloning cDNA for dihydrofolate reductase, the target enzyme for drug methotrexate (MTX), from a highly MTX-resistant mouse 3T6 subline. DNA sequencing showed that the mutation involved a single substitution of a guanine for a thymine, which resulted in the substitution of an arginine or a leucine at position 22 of the protein.

Point mutation is not the sole genetic change that can lead to the development of resistance to a drug or inhibitor. Further understanding of the molecular nature of resistance to purine analogues has come from the cloning of structural genes for the enzymes that may be altered during the development of resistance. Analysis has been made of changes in the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene that can lead to resistance to 6-mercaptopurine and 6- thioguanine (purine analogues). The cloned cDNA sequence has been used as a probe to identify changes in restriction fragments in this gene in resistance mutations.

Fuscoe *et al.* (1983) found that the reduced HGPRT activity of the two mutants derived from hamster cell cultures involved deletions of large sections of the gene, whereas results obtained from 17 other mutants of spontaneous and UV-induced

TABLE 3 Drug resistant mutants (Stark and Whal, 1984)

Drug(s)	Enzyme or binding protein	References
5-Fluorodeoxyuridine	Thymidylate synthetase	Rossana *et al.* (1982)
α-Methyl- orα-difluoromethyl-ornithine	Ornithine decarboxylase	Choi and Scheffler (1981)
Hydroxyurea or deoxynucleotides	Ribonucleotide reductase	Ashman and Davidson (1981)
Aphidicolin	DNA polymerase α *(Drosophila)*	Sugino and Nakayama (1980)
Aphidicolin	Ribonucleotide reductase	Sabourin *et al.* (1981)
Adenine and coformycin	AMP deaminase	Debatisse *et al.* (1982)
Albizziin or -aspartyl hydroxamate	Asparagine synthetase	Andrulis *et al.* (1983)
Mycophenolic acid	IMP-5'-dehydrogenase	Huberman *et al.* (1981)

TABLE 3 (continue)

Tunicamycin	N-Acetyl glucosaminyl transferase	Criscuolo and Krag (1982)
Borrelidin	Threonyl-tRNA synthetase	Gantt *et al.* (1981)
Canavanine	Argininosuccinate synthetase	Su *et al.* (1981)
Methotrexate	DHFR (two distinct genes)	Lewis *et al* (1982)
Methotrexate	Bifunctional thymidylate synthetasedihydrofolate *(Leishmania)*	Corderre *et al.* (1983)
PALA	CAD	Wahl *et al.* (1979)
Cadmium	Metallothionein I	Gick and McCarty (1982)
6-Azauridine or pyrazofurin	UMP Synthetase	Suttle (1983)
Adenosine, alanosine, and deoxycoformycin	Adenosine deaminase	Hoffee *et al.* (1982)
Hypoxanthine,	Mutant hypoxanthinegua-	Brennard *et al.*

TABLE 3 (continue)

aminopterin, and thymidine ("HAT")	nine phosphoribosyl transferases	(1982)
Compactin	3-Hydroxy-3-methylglutaryl coenzyme A reductase	Hardeman *et al.* (1983)
Methionine sulfoximine	Glutamine synthetase	Sanders and Wilson (1984)
Histidinal	Histidyl-tRNA synthase	
6-Thioguanine	Hypoxanthine-guanine	O'Neill *et al.* (1977)
8-Azaquanine	Phosphoribosyltransferase	Fox *et al.* (1976)
Ouabian	Ouabian-binding subunit of Na^+K^+-ATPase	Cole and Arlett (1984)
2, 6-diaminopurine	Adenine phosphoribosyltransferase	Brown *et al.* (1983)
Tubercidin	Adenosine kinase	Rabin and Gottesman (1979)
5-Bromodeoxyuridine	Thymidine Kinase	Clive *et al.* (1972)

mutants have been shown to be consistent with being point mutations. In addition, Turner *et al.* (1985) found that 57% of thioguanine-resistant human lymphocytes showed either deletions or amplifications of the HGPRT gene that were not detectable cytogenetically. Thus it has been shown that a variety of DNA changes can be involved and that different classes of genetic changes can be varied by different treatments.

Evidence for complex genetic changes being involved in purine analogue resistance came from an early study by Fox and Radacic (1978) which showed that selection for resistance to a single high dose of purine analogues produced stable resistant rodent cell lines; whereas selection for resistance following prolonged exposure to low levels of purine analogues led to very unstable resistant clones. This implies that the first procedure involved point mutation. Studies on the mutation in the gene for adenine phosphoribosyltransferase (APRT) has shown that it requires the loss of two copies of the gene. The development of resistance can be a two-stage process. A high-frequency event (gene inactivation or deletion) can occur either preceded by or followed by a low frequency event namely base substitution (Simon and Taylor, 1983).

Repeated duplication of regions of DNA can lead to the production of multiple copies of certain genes and their flanking DNA sequences which is known as gene amplification (Stark and Wahl, 1984). The relative importance of gene resistance probably varies between drugs. Amplification of genes confering resistance to methotrexate and 5-fluorouracil has been found in tumors and cell cultures (Clark *et al.*, 1987). It was proposed by Schimke *et al.* (1986) that overproduction results from the accumulation of an increased capacity for the initiation of DNA synthesis in cells where DNA synthesis is partially inhibited but RNA and protein synthesis

can continue. It therefore follows that drugs or treatments that preferentially inhibit DNA synthesis relative to RNA and protein synthesis are more likely to result in amplification events leading to resistance phenomena.

The rate of spontaneous mutation frequency for drug resistant mutants is in the order of 10^{-5}-10^{-11} eg 1-5x10^{-6} for HGPRT deficient (O'Neill *et al.*, 1977), 5x10^{-11} for thymidine kinase deficient (Clive *et al.*, 1972), 3x10^{-6} for adenosine phosphoribosyltransferase, and 5x10^{-5} for dihydrofolate reductase (Brown *et al.*, 1983)

INTRODUCTION TO THE PROJECT

A variety of evidence suggests that some of the biological functions of ADPR transferase are associated with DNA repair, gene expression and differentiation. Such conclusions are based on results involving ADPR transferase inhibitors which are complicated by their other metabolic effects in mammalian cells. The elucidation of its function in biological systems would be greatly facilitated by the availability of cell lines with a deficiency in this enzyme. The identification of mutant cell lines defective in a particular enzyme activity has been a useful approach in identifying the function of some enzymes. Such an approach was demonstrated by Cleaver (1968) who observed that fibroblasts in culture derived from the skin of a human patient with the disease *Xeroderma pigmentosum* were defective in excision repair following exposure to UV irradiation. This resulted in the availability of mutant cells defective in their response to DNA damage.

The principal reason why mutants provide so powerful an approach to understanding cell function is that they allow a comparison of the abnormal state with the normal state under conditions of an isogenic background. By permitting an examination of the metabolic consequences of altering one particular component of the cell, a mutant serves as a tool for probing functional relationships. Mutants may therefore be used as analogues to that of inhibitory drugs with the advantage that their specificity of action is much more certain.

This project has aimed at isolating spontaneous mutant L1210 cells resistance to the cytotoxicity of 3-acetamidobenzamide (3-AAB), an inhibitor of ADPR transferase. At the concentration used (5 mM), 3-AAB was shown to be too toxic to wild-type, hence there was a possibility that some mutants would arise as a result of

alterations in poly (ADP-ribose) metabolism.

The mutant cells were isolated using a single-step method in which cells were plated in large numbers in a soft agar plate containing 5 mM 3-AAB. The individual clones grown in soft agar were picked and grown in suspension medium containing 5 mM 3-AAB. Various assays (as described in following chapters) were carried out on 12 mutant cell lines together with their wild-type cells. Their properties were then compared from which conclusions were subsequently drawn.

CHAPTER 2

MATERIALS

CHAPTER 2

MATERIALS

2.1 INHIBITORS, THEIR ACID ANALOGUES, AND CYTOTOXIC AGENT

The following were synthesized by B. Hunt in this laboratory : 3-acetamidobenzamide, 3-aminobenzamide and 3-acetamidobenzoic acid, whilst 3-nitrobenzamide, 3-aminobenzoic acid, 3- nitrobenzoic acid and dimethyl sulphate (DMS) were purchased from Aldrich Chemical Company Ltd. U.K.

2.2 TISSUE CULTURE MATERIALS

Mouse lymphoma cells, strain L1210, used throughout this study, were obtained from Flow Laboratories, Irvine Scotland. The following components for the growth media were also obtained from the same source : RPMI 1640 growth medium (10 x concentration), L-glutamine (200 mM), penicillin-streptomycin (500 IU/ml and 5000 Ug/ml respectively), donor calf serum (mycoplasma and virus screened), sodium bicarbonate (7.5%) solution, and trypan blue (0.5% w/v in 0.85% saline), sterile petri dishes of 6 cm in diameter were purchased from Sterilin, Scotland.

2.3 RADIOACTIVE LIGANDS AND RADIOACTIVITY COUNTING

[^{3}H] NAD was prepared from [^{3}H]-ATP (Radiochemical centre Amersham, Bucks) by Dr W. J. D. whish according to the method of Ohtsu and Nishizuke (1971). [^{3}H]-NAD was prepared in ethanol with a final specific activity of 23 mCi/μmole and radioactive concentration of 1 mCi/ml.

[Carbonyl - ^{14}C] nicotinamide (53 mCi/mole made up to 10 μCi/ml with 100% ethanol was obtained from Radiochemical Centre Amersham, Buckinghamshire U.K.

[^{3}H] 3-acetamidobenzamide (5.85 nCi/mole) was prepared in this laboratory by Dr Whish and B. Hunt in 100% ethanol (117 nCi/ml).

Optiphase was purchased from BDH Chemical Ltd. 3MM filter paper disc and glass fibre disc (GFC) were supplied by Whatman Ltd, Maidstone, Kent, U.K.

2.4. THIN LAYER CHROMATOGRAPHY

PEI cellulose thin layer sheets (20 cm x 20 cm) were obtained from Camlab, Cambridge. Standard marker for NAD was purchased from Boehringer Corporation, Lewis, Sussex.

CHAPTER 3

METHODS

CHAPTER 3

METHODS

3.1 STERILE TECHNIQUE

All equipment and non-sterile components of growth medium such as bottles, tubes, plugged pasteur pipettes, plastic tips and double glass distilled water, were sterilized by autoclaving at 120 deg.C for 20 min. The pipettes for measuring volume were plugged with cotton wool and sterilized overnight in pipette canisters in an oven at 160 deg.C.

3.2 PREPARATION OF RPMI GROWTH MEDIUM (pH7.4)

Normal RPMI 1640 complete growth medium was prepared by diluting RPMI 1640 10 x concentration with sterile double glass distilled water, 10% (v/v) calf serum, 2 mM glutamine, 0.2% (v/v) sodium bicarbonate and 100 IU each of penicillin and streptomycin, stored at 4 deg.C and used within one month. Medium used for the cloning assay containing 0.3% (w/v) of agar was prepared by the same method as before and kept at 37 deg.C.

3.3 CELL CULTURE

Mouse L1210 cells were usually stored in liquid nitrogen. When required, these were thawed at 37 deg.C in a water bath and suspended in complete RPMI growth medium to a cell density of about $2x10^5$ cell/ml. After 18h, the dimethyl sulphoxide remaining in the medium was removed by carefully aspirating the

medium to low level leaving cells undisturbed and fresh medium was added. The growth of such culture was checked periodically to ensure that the doubling time was 12-13 h. All cell counting was performed using a modified Neubauer haemocytometer, which allows frequent microscopic examination of cell morphology. A minimum of 40 cells were counted on each set of nine chambers and the reproducibility of cell counting was shown to be ±10%. L1210 cells enter the stationary phase (Go) at about $1x10^6$ cells/ml. The cell line was maintained by subculturing to a cell density of not less than $5x10^4$ cells/ml. Cells from cultures in mid-log phase were used for all experiments.

3.4.1 ISOLATION OF MUTANT L1210 CELLS RESISTANT TO CYTOTOXICITY OF 5 mM 3-ACETAMIDOBENZAMIDE (3-AAB)

Mutant L1210 cells were selected in a stringent system by the ability to form colonies in soft agar containing 5 mM 3-AAB. About $1.0\text{-}1.5x10^6$ cells were seeded in 8-10 ml agar medium in 6 cm petri dishes. For each set of isolation experiment about 20 (or more) petri dishes were prepared. These petri dishes were incubated for at least 4 weeks at 37 deg.C in a humidified atmosphere of 5% (v/v) CO_2/air (see chapter 4 section 4.1.4). 5 mM 3-AAB was present in agar medium throughout the incubation time.

Mutation frequencies are expressed as per 10^7 viable cells plated. Calculated from the equation below.

$$\frac{\text{No of mutants}}{\text{No of cells seeded x Cloning efficiency}}$$

3.4.2 THE PROCESS OF FREEZING MUTANT CELL LINES IN LIQUID NITROGEN

Mutant cells were grown in media containing 5 mM 3-acetamide- benzamide (selective medium) to obtain a large population of cells (about 250-300 ml). Cells were collected during late logarithmic growth curves (early stationary phase). This was done by using a sterile pasteur pipette to remove the supernatant media, leaving the layer of cells on the bottom undisturbed. The supernatant media was removed so that a high density of cells ($2x10^6$ cells/ml) was achieved. The bottles of the cells were then placed on ice 5 min prior to adding 1 ml of DMSO for every 10 ml of cells. Aliquots of 2 ml of cells were dispensed into sterile tubes. The tubes were cooled slowly over liquid nitrogen in a special container overnight. The process of slow cooling could also be achieved by insulating the tubes with polystyrene (2 inches thick) then placing them at -70 deg.C overnight. The following day, all the tubes were placed in liquid nitrogen storage.

3.4.3 THE PROCESS OF GROWING MUTANT CELLS FROM LIQUID NITROGEN STORAGE

Mutant cell lines stored in liquid nitrogen, were thawed at 37 deg.C inawater bath and suspended in complete RPMI growth medium to a cell density of $2x10^5$ cells/ml. After 18 h cells were collected by centrifugation (1000 rpm for 5 min) using a bench centrifuge and selective medium was added. This procedure was adopted so that DMSO was removed and mutant cells were maintained in selective medium as stock cells ready to be prepared for various experiments. A stock of mutant cells were checked periodically and routinely subcultured to $5x10^4$ cells/ml when they reached the stationary phase. The stock of mutant cells were replaced

after 3 months by thawing another tube of cells obtained from liquid nitrogen storage.

3.4.4 PREPARATION OF MUTANT CELLS FOR VARIOUS EXPERIMENTS

Mutant cell lines were prepared for various experiments from a stock of cells (from mutant cells kept in medium containing 5 mM 3-AAB), by washing the cells 4 times with 15 ml of warm normal RPMI growth medium. This washing was to ensure that 3- AAB was removed. After washing, mutant cells were grown in normal RPMI medium and were subcultured at $5x10^4$ cells/ml ready for various experiments. All mutant cells grown in medium free of 3-AAB were used for 10 days then discarded. Mutant cells were always used during mid-log phase for all experiments.

3.4.5 ESTIMATION OF SURVIVAL OF L1210 AND MUTANT CELL LINES IN AGAR CONTAINING VARIOUS CONCENTRATIONS OF 3-AAB

An aliquot of 20 µl (100 cells) from the final dilution was plated on each petri dish with 8 ml of complete RPMI 1640 medium supplemented with 0.3% agar and the chosen concentration of 3- AAB was present in the agar medium throughout the incubation time. A high number of cells (>100 cells) were plated if the concentration chosen was too toxic to allow less than 1% of cells to survive. Five petri dishes were prepared for each treatment group and they were incubated for 10-14 days for wild- type cells and 22-27 days for mutant cells.

For those treatment groups requiring pretreatment with 50 µM dimethylsulphate (DMS) for 1 h, 3-AAB (0.5 mM) was added to cells at least 10 min prior to the

addition of DMS. 3-AAB was present throughout the dilution procedure and during the incubation time. The washing procedure was regarded as unnecessary due to the high cell density (10^6 cells/ml) used and the subsequent small aliquot (about $5x10^4$ cells) of cell suspension required for the serial dilutions. Furthermore, the half-life of DMS is short (about 10-15 min) thus the amount of residual DMS was considered to be negligible.

3.5 CELL VIABILITY

Cell viability was determined using 0.2% trypan blue in saline, an equal volume of medium was usually added. The cells were examined under the microscope. Dead cells or permeable cells (permeabilised cells) were stained blue, while viable cells remained unstained.

3.6 PREPARATION OF INHIBITORS AND DMS

Stock solutions of 3-acetamidobenzamide and 3-nitrobenzamide at concentrations above 10 mM (left at room temperature) were heated to ensure complete solubilization; up to 24 mM they were in solution at 37 deg.C. Both inhibitors are very stable when heated, hence stock solutions were prepared by dissolving in double glass distilled water and sterilized by autoclaving at 15 lb/in for 20 min.

3-Aminobenzamide is readily soluble in warm double glass distilled water up to 100 mM. Stock solutions were sterilized using a microflow filter, pore size 0.22 u and stored at 4 deg.C.

The three acid analogues (3-acetamidobenzoic acid, 3- nitrobenzoic acid and 3-

aminobenzoic acid) are insoluble in water, thus sodium hydroxide was added and the solution adjusted to pH 7.4. Stock solutions were prepared and filter- sterilized using a microflow filter.

DMS is a liquid at room temperature and does not require sterilization. Solution of DMS in absolute ethanol was prepared immediately before use.

3.7 SCINTILLATION COUNTING

All radioactive samples were counted in Packard Tri-Carb liquid scintillation spectrometer. Optiphase was used as scintillant; for most non-aqueous samples, 3 ml of optiphase solution was used but for aqueous samples, 9 parts of optiphase was used for every 1 part of aqueous sample.

3.8 MEASUREMENT OF ACID INSOLUBLE RADIOACTIVITY

Acid-insoluble radioactivity was measured by applying the reaction mixture (up to 25 µl) to whatman 2.4 cm filter paper discs (presoaked in 20% (w/v) TCA in diethylether for 15 min). The discs were washed four times in 400 ml of ice cold 5% TCA (w/v) for 15 min each and once in 95% ethanol. After drying, the radioactivity was counted. Counting efficiency was 85-90% for [^{14}C] and 55-60% for [^{3}H].

3.9 THIN LAYER CHROMATOGRAPHY (t.l.c)

Thin layer chromatography was performed using PEI cellulose. Before use, it was first soaked in 2M NaCl for 30 min and then in distilled water for at least 30 min

and left to dry at room temperature. Standard NAD was made up in 50% ethenol at a final concentration of 5-10 mg/ml. Samples (about 20 µl) were applied to the origin (2 cm from the bottom of the plate) using capillary tubes and dried under a stream of cold air. The spots on the t.l.c. plate were left to develop in a butanol system for 3-5 h. In this system, Butanol: Water: Methanol: Ammonia in the ratio 60:20:20:1 was used to achieve a good separation of nicotinamide (RF=0.71) and the pyridine nucleotides NAD, NADH and NADP (RF=0) which remained at the origin. After developing, the plates were then visualized under an ultraviolet (UV) light with emission at 250-280 nm. For radioactive counting, the sheet was divided into 1 cm strips, 1 ml of 0.5 M percholic acid was added and incubated in scintillation vials at 90 deg.C for 30 min. This procedure hydrolyses and solubilises the radioactive adenine group from the rest of the nucleoside/nucleotide. Samples were left to cool and then counted.

3.10 CELL PERMEABILIZATION

The cell permeabilisation method was essentially that of Durkacz *et al* (1980a). Cells were chilled to 4 deg.C and centrifuged in the cold for 5 min at 1500 rpm using MSE bench centrifuge. They were washed once with ice-cold Puck's saline (containing 400 mg/l KH_2PO_4 , 350 mg/l $NaHCO_3$, 8 mg/l NaCl and 1 g/l glucose at pH 7.4). The pellet was resuspended in cold hypotonic buffer (containing 9 mM HEPES, 4.5% (w/v) dextran, 1 mM EGTA, 4.5 mM $MgCl_2$, 5 mM DTT at pH 7.8) to a final density of $3\text{-}5x10^7$ cells/ml and were left at 4 deg.C for 50 min. Finally, this concentrated cell suspension was diluted 10 times by the addition of cold isotonic buffer (containing 40 mM HEPES, 130 mM KCl, 4% (w/v) dextran, 225 mM sucrose, 2 mM EGTA, 2.3 mM $MgCl_2$ and 2.5 mM DTT). The efficiency of permeabilization was determined by trypan blue exclusion.

3.11 TRANSPORATION OF 3-ACETAMIDOBENZAMIDE (3-AAB) INTO THE CELLS

Cultures of wild-type L1210 and mutant cells (3 ml) at $5x10^6$ cells/ml were incubated with 15 µl of [^{3}H] 3-AAB (117 nCi/ml). At various times, 200 µl sample ($1x10^6$ cells) were filtered on to Millipore GFC disc (prewashed with 3 ml ice cold PBS). The discs were then washed with 3x10 ml of cold PBS, dried at room temperature and the radioactivity counted.

3.12 DNA ESTIMATION

The Burton assay was used for measuring DNA; it is a colorimetric procedure for measuring the deoxyribose moiety of DNA (Burton, 1956). The procedure was divided into 2 steps. For the first step; whole cells containing lipids ($2x10^6$ cells) were washed, first with 0.2 M PCA in 50% ethanol and 50% distilled water, mixed well and left on ice for 15 min. The cell pellet was collected by centrifugation for 5 min at 1500 rpm using an MSE bench centrifuge. It was then washed with 1 ml of ethanol-ether (3:1, v/v), left for 10 min at 70 deg.C then centrifuged as above. Finally, the pellet was mixed with 1 ml of 95% ethanol, vortexed and centrifuged to discard the supertanant.

Second step: The pellet was treated with 1 ml of 0.5 M PCA and hydrolyzed for 70 min at 70 deg.C, cooled on ice, centrifuged and the supernatants were collected into marked tubes. For every 1 volume of supernatant added, 2 volumes of diphenylamine reagent. It was prepared using 1.5 g of diphenylamine in 100 ml glacial acetic acid and 1.5 ml of (98-100%) H_2SO_4, just before used 0.5 ml of acetaldehyde stock solution (2 ml of acetaldehyde in 100 ml distilled water) was

added to it. The solution was mixed and incubated at 30 deg.C for 18 h. The absorbances at 595 and 650 nm, using 0 µg/ml standard as a blank, were read from the spectrophotometer. The unknown DNA concentrations of the cells were read off from the standard curve of known DNA concentration.

For the standard curve of known DNA concentrations, 0.5 ml DNA stock solutions (8.5, 17, 42.5, 85 and 170 µg) in 1 ml of distilled water was mixed with 0.5 ml of 1 M PCA, then assayed according to the step two above. A standard curve was obtained by plotting the absorbance at 595 nm minus absorbance at 650 nm as a function of the initial DNA concentration.

CHAPTER 4

ISOLATION OF MUTANT L1210 CELLS RESISTANT TO 5 mM 3-ACETAMIDOBENZAMIDE AND THEIR CHARACTERIZATIONS

CHAPTER 4

4.1 PROCEDURE FOR THE ISOLATION OF MUTANT CELLS RESISTANT TO AN INHIBITOR OF ADPR TRANSFERASE

4.1.1 Cell line

Mouse lymphoma L1210 cells were used for this investigation as they have been routinely used in this laboratory for the study of various aspects of ADP-ribosylation. This cell line has been adopted to grow in culture and it has characteristics that are of general interest to investigators. The cells grow quickly with a generation time of approximately 12-13 h and they are easy to handle as they grow in suspension culture. The cells do not adhere to the culture vessel and they only associate loosely with each other. This is an advantage as the cells do not need to be trypsinized. This has greatly simplified maintenance of cultures and the measurement of cell number and growth rate.

L1210 cells can form single - cell derived colonies when immobilized in medium made semisolid with agar. This property is defined as plating efficiency. It measures the number of colonies of cells (each colony being derived from one cell) that form from dilute concentrations of cells plated in agar medium (usually 100-200 cells). This is calculated as:

$$\frac{\text{No of colonies isolated} \times 100}{\text{No of cells seeded}}$$

The plating efficiency for L1210 cells determined in this laboratory is 72±4.9% (50

determinations). This high plating efficiency has proven invaluable in quantitating the effect of drugs on cell viability in the isolation of drug-resistant mutants. In many established cell cultures (and most primary cell cultures) the plating efficiency is too low. It is often less than 1% or as low as 0.001% (Ham, 1964; 1965). It was found that in the development of cloning techniques cells leak nutrient into the medium and secrete chemical messengers that are required for survival and growth (Samford *et al.*, 1963; Ham, 1963b) hence, some cell lines do not give a good plating efficiency.

4.1.2 Inhibitor of ADPR transferase used

3-Acetamidobenzamide (3-AAB) was chosen as it has been shown to be the most potent of the ADPR transferase inhibitors causing 98% inhibition of the enzyme from calf thymus at 1 mM. Moreover, 3-AAB is 6-8 times more effective as ADPR transferase than is 3-aminobenzamide (Purnell and Whish, 1980).

4.1.3 Dose of inhibitor

A suitable concentration of inhibitor was chosen for mutant selection. It was suggested by Rabin and Gottesman (1979) that the 2 x concentration of drug which allows survival of 10% of the original population (LD_{10}) is a suitable concentration for selection purposes. It was found that the killing of the original population was substantial at such concentration.

The dose response of wild-type cells to a potential selective inhibitor could be measured either in terms of growth rate or plating efficiency in the presence of inhibitor. Plating efficiency is preferable because it permits an evaluation of

population heterogeneity throughout the dose range and is also a more sensitive indicator of the minimum dose necessary to kill the wild-type cells and to discern any unusually resistant clones. The percentage survival of L1210 cells in various concentrations of 3-acetamidobenzamide (3-AAB) is determined in agar medium and is shown in figure 5. It is expressed as percentage survival of the control, calculated from equation below.

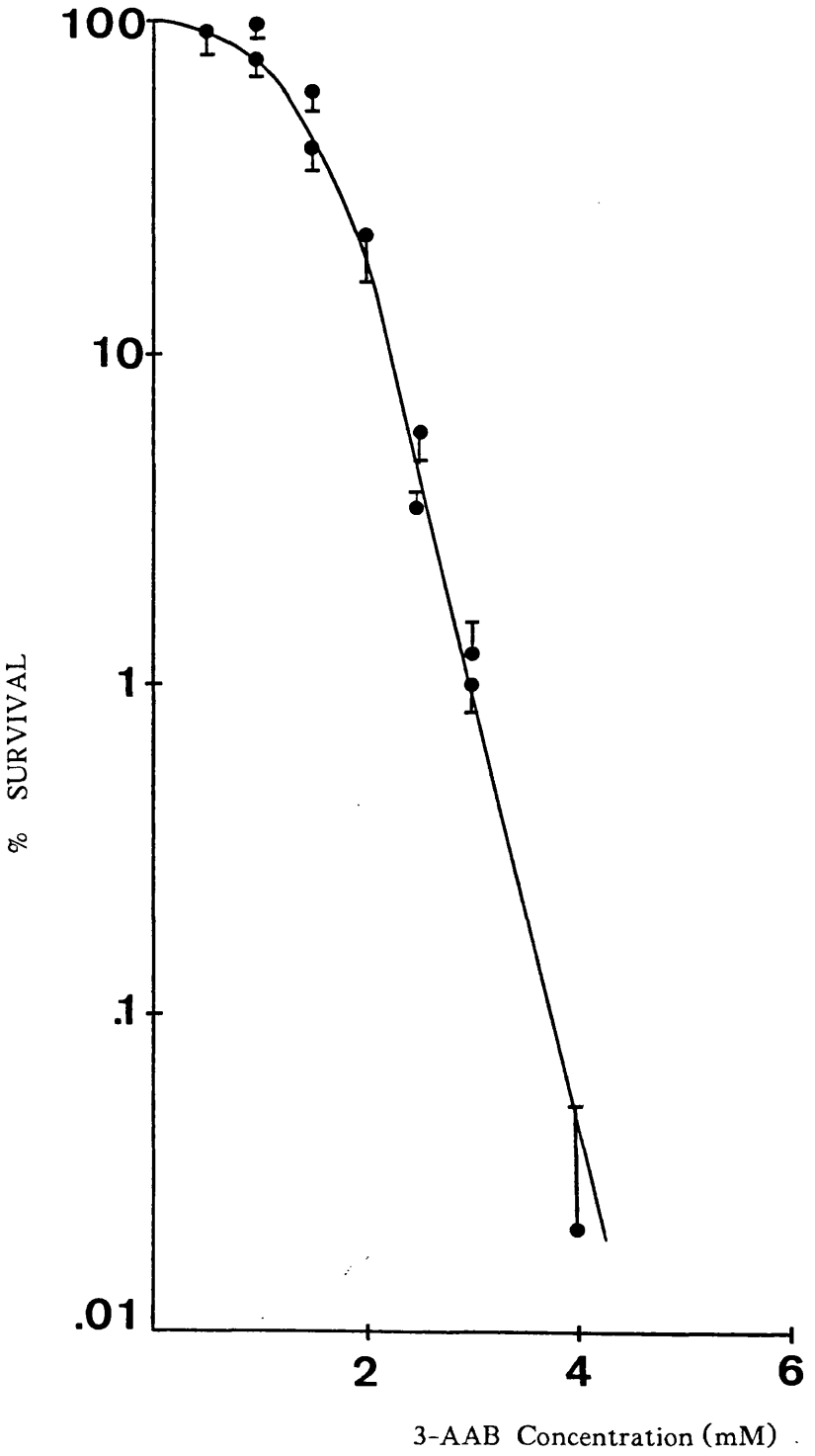
$$\frac{\text{No of cells grown into colonies in 3-AAB}}{\text{No of cells grown into colonies in the absence of 3-AAB}} \times 100$$

The concentration of 3-AAB which allows 10% of cells to survive is about 2.5 mM. The concentration of 5 mM (2 x 2.5 mM) 3-AAB was therefore chosen to isolate the mutant cells resistant to 3-AAB.

4.1.4 Methodology of developments for the isolation of mutant cells during the long incubation period in CO_2/air

A large population of wild-type cells were exposed to 5 mM 3- acetamidobenzamide in agar medium for the period of at least 4 weeks. This period was considered to be quite long compared to only 10-14 days for a normal single cell which grows into a colony in a normal agar medium RPMI 1640. When this work was initiated two major problems threatened the opportunity of obtaining mutant cells. The biggest problem was the medium evaporating during the long incubation period. Eventhough cells in petri dishes were maintained in a humidified CO_2/air incubator, the humidity generated in the incubator was not sufficient to prevent drying of the agar medium . It was observed that the agar medium was reduced to 3/4 of its original volume by the end of the third week. This means that populations of cells

FIGURE 5 Cytotoxicity of 3-AAB on L1210 cells treated continuously in soft agar.



Diluted cells (100 or more) were plated on to 6 cm petri dish containing various concentrations of 3-AAB. After 3 weeks colonies were counted and calculated as percentage survival from equation below (5 petri dishes per each data point).

$$\frac{\text{No of colonies in the presence of 3-AAB} \times 100}{\text{No of colonies in the absence of 3-AAB}}$$

were exposed to the increasing concentrations of 3-AAB, which is so toxic that it will not permit even the mutant cells to develop into colonies. The second problem was the mycoplasma or fungal contamination found in the environment, which affected the cloning procedures.

A method was therefore developed to circumvent medium evaporation and to minimize the risk of contamination. A square plastic box, easily obtainable from any supermarket store, of 25 (length) x 8 (height) cm was used as mini incubator when placed inside the CO_2/air incubator. Four holes of about 2.5 cm in diameter were made at each side of the square box. The holes were sealed with kitchen cling film, to act as a permeable membrane for CO_2.

Cells grown in suspension or agar medium, inside the box with the lid closed tight, have been found to behave as cells grown outside the box (with the same generation times). This demonstrated that CO_2 in the incubator is passing through the cling film into the box. Sterile water was placed at the bottom of the box to provide humidity which prevents the agar medium from becoming dry during the incubation period. It was observed that medium evaparation had not occured even at the end of 2-3 months. Washing the inside of the box with 75% ethanol before use, with the lid closed very tightly, helped to minimize contamination. Another advantage with using these boxes is that it has reduced space shortage inside the CO_2/air incubator as boxes can be placed on top of one another. This allows large experiments involving the use of many petri dishes to be performed. Plastic boxes made for the purpose of isolation mutant cells are still being used in this laboratory to grow various cell lines for various experiments.

4.1.5 Cloning mutant cells resistant to 5 mM 3- acetamidobenzamide (3-AAB)

Wild-type L1210 cells were grown in suspension medium in glass tissue culture bottles. For routine subculturing, cells were counted using a haemocytometer to estimate cell numbers. This provided a visual check on the state of the cells. Cells used were always in mid-log phase (5-7 x 10^5 cells/ml) as most of them were healthy and active. Cells were collected by centrifugation and about 1.00-1.5 x 10^6 cells were plated in 6 cm petri dish containing 8-10 ml 0.3% agar medium supplemented with 5 mM 3-AAB. Once all cells were plated on to petri dishes, which were varied according to total cells used (for example 10 petri dishes for 10 x 10^6 cells), they were put inside the plastic boxes and placed in CO_2 incubator.

It is sometimes necessary to treat cells with a mutagen in order to obtain mutants in significant numbers (Kao and Puck, 1967). In this investigation mutagens (eg DMS, MNNG) were not used as ADPR transferase activity is stimulated by such agents (see chapter 1 section 1.1.9). Such procedures might interfere with the isolation of mutants which are potentially ADPR transferase deficient.

Cloning mutant cells in soft agar plating allows the individual cells which are resistant to the cytotoxic effect of 3-AAB to divide and form colonies large enough to be seen by the naked eye. The normal soft agar plating of L1210 cells produces colonies of 1 or 2 mm in diameter within 10-14 days, but mutant cells treated with 5 mM 3-AAB required at least 4 weeks before the colonies had grown enough to be picked and maintained in suspension culture in the presence of 5 mM 3-AAB. This selective pressure was constantly present in the population of mutant cells to prevent them being overgrown by revertant cells during continuous subculture.

The total number of cells used for various cloning procedures is shown in table 4. It also shows the number of colonies obtained in each cloning assay. Some colonies

failed to proliferate in suspension medium. Without further investigation, it is not easy to explain the reasons for such phenomena. Mutant frequency per survival is calculated from the equation below.

$$\frac{\text{Total colonies observed in 5 mM 3-AAB}}{\text{Total number of cells plated in 5 mM 3-AAB x Plating efficiency}}$$

Out of 22 colonies which had proliferated in suspension medium only 12 clones were investigated. This is because each mutant requires a good deal of time for its investigation. It was considered that 12 mutants were sufficient for a preliminary investigation.

The 12 mutant clones were re-cloned again in the agar medium containing 5 mM 3-AAB. The re-cloning was to ensure separation of cells which may have clumped together at the time of the first cloning (a colony which did not arise from a single cell). This procedure ensured that each mutant clone tested was derived from single cell.

4.1.6 Re-cloning of mutant cells in soft agar plating

Mutant cell lines have been established in suspension medium in the presence of 5 mM 3-AAB and have been subcultured 2- 3 times. The sub-culturing was done every 3-4 days at $5x10^4$ cell/ml. Mutant cells were diluted and 100 cells were plated in soft agar containing 5 mM 3-AAB. After a period of 4 weeks, 1 colony from each mutant cell line was picked and recloned again for the third time. The 2 consecutive clonings were performed so that their resistance to 3-AAB could be tested in terms of plating efficiency in agar cloning assay. Results obtained for the 2

consecutive cloning assays are shown in table 5.

DISCUSSION

The apparent mutation frequency per survival of L1210 cells, which result in resistance to 5 mM 3-AAB is in the range of 1- 3 x 10^{-7} . Other spontaneous mutation frequencies are in the range of 10^{-5} -10^{-11} (see chapter 1 section 2.3 and 2.4).

Plating efficiency of mutant cells for the first re-cloning is quite low (see table 5). Colonies obtained from 100 cells plated are in the range of 5-30 colonies. A higher number of colonies were obtained from the second re-cloning in which 50 colonies or more per petri dish was achieved. This suggests that when mutant cells were maintained under selective pressure, a progression to higher level of resistance occurs.

Low plating efficiency from the first re-cloning suggests that some mutant cells from various lines are unstable. This phenomenon was recognized earlier (Nature (London), New Biol 237, 98 (1972)).

TABLE 4 Clones of mutant L1210 cells resistant to 5 mM 3- acetamidobenzamide (3-AAB)

Number of cloning assays	Total cells in each assay x 10^7 cells	No of colonies isolated per assay	Mutation frequency per survival per 10^{-7}	No of colonies grown in suspension medium 5 mM 3-AAB
1	2.2	2	1.28	2
2	2.7	4	2.10	4
3	4.7	4	1.20	2
4	5.2	12	3.29	3
5	6.5	9	2.00	4
6	4.5	3	0.95	3
7	10.0	11	1.57	1
8	6.2	9	2.07	1
9	6.7	11	2.34	2
Total	48.7	65		22

A large population of wild-type L1210 cells (about $1x10^6$ cells) were plated onto 6 cm petri dish, containing agar medium supplemented with 5 mM 3-AAB (See chapter 3.4.1). The number of mutant clones obtained in each stage of cloning assay, mutation frequency and number of mutant clones which survived in suspension medium in the presence of 5 mM 3-AAB are listed.

TABLE 5 Re-cloning of mutant cell lines in 5 mM 3 - acetamidobenzamide (3-AAB)

Mutant cell line number	First re-cloning number of colonies from 100 cells plated (5 results per data point)	Second re-cloning number of colonies from 100 cells plated (5 results per data point)
3	9.0 ± 3.0	78.8 ± 2.0
4	10.0 ± 0.5	60.3 ± 5.7
5	11.0 ± 1.7	70.3 ± 4.3
6	16.3 ± 2.8	72.6 ± 2.9
7	32.0 ± 1.7	75.1 ± 1.4
9	11.3 ± 3.1	54.0 ± 5.2
10	10.6 ± 1.5	79.0 ± 3.6
12	12.5 ± 5.0	90.3 ± 9.0
13	26.0 ± 3.8	89.3 ± 13.0
14	33.0 ± 2.0	76.0 ± 3.6
15	16.4 ± 4.0	31.8 ± 1.0
16	5.3 ± 1.2	30.0 ± 2.5

Mutant cell lines isolated from wild-type cells (see 4.1.5) grown in suspension medium containing 5 mM 3-AAB were diluted and plated on to 6 cm petri dish. After 4 weeks, mutant colonies picked from 1st re-cloning were re-cloned as before (see 4.1.6).

4.2 CHARACTERIZATIONS OF MUTANT CELL LINES

One of the clones from the second re-cloning was isolated for each mutant cell line then regrown in suspension medium containing 5 mM 3-AAB. The following characters were analysed and compared with their wild-type L1210 cells.

4.2.1 Growth rates of mutant cell lines in the presence and absence of selective pressure (5 mM 3-AAB)

To determine the growth rates, 5 ml of wild-type and mutant cell lines were suspended in culture medium at 5 x 10^4 cells/ml to determine cell density (cells/ml) over a period of 98 h. At time intervals of 12 h, cell density was determined with a haemocytometer using a Zeiss binocular microscope. Results were summarized and presented in tables 6 (a&b) and 7.

DISCUSSION

Figure 7 shows the growth curves of mutant 3 and wild-type cell lines in the absence and presence of 5 mM 3-AAB. Growth curves of other mutant cell lines are not shown as they are similar. The doubling times and maximum cell densities at the stationary phase are summarized in table 6 a, b. Table 7 shows the ratio of doubling time of wild-type to mutant cell lines and the ratio of the doubling times of mutants, within the same cell line, in the presence and absence of 5 mM 3-AAB.

When selective pressure is absent, wild-type cells started to enter early stationary phase at about 1 x 10^6 cells/ml whereas mutant cells start to enter early stationary phase at about 3- 4.5 x 10^5 cells/ml. (See table 6a). Furthermore, wild-type cells

TABLE 6 a. Comparison of the doubling time of wild-type and mutant cell lines grown in RPMI 1640 medium (no 3-AAB) and the maximum cell density (cells/ml) at stationary phase. (see also figure 6 a&b)

Cell lines	Doubling time (h)	Maximum cell density (cells/ml)	Number of experiments
Wild-type	12 ± 2	100 ± 12	10
Mutant 3	29 ± 1	41 ± 7	9
4	23 ± 1	38 ± 6	6
5	24 ± 2	37 ± 3	6
6	29 ± 3	32 ± 6	6
7	20 ± 1	46 ± 5	6
9	23 ± 1	37 ± 2	6
10	24 ± 2	40 ± 4	6
12	26 ± 2	41 ± 5	6
13	20 ± 1	38 ± 5	6
14	24 ± 2	34 ± 3	6
15	20 ± 2	33 ± 4	6
16	22 ± 2	35 ± 3	6

Cells (at $5x10^4$ cells/ml) were grown in RPMI 1640 medium over period of 96 h, at time intervals of 12 h the cell density was determined. Cell cycle doubling times were determined from slopes of growth curves, calculated from least square regression analysis of the data.

TABLE 6 b. Comparison of doubling time of wild-type and mutant cell lines grown in RPMI 1640 medium containing 5mM 3-AAB and the maximum cell density (cells/ml) at stationary phase (see also figure 6 a & b)

Cell lines	Doubling time (h)	Maximum cell density (cells/ml)	Number of experiments
Wild-type	0	N.P.	3
Mutant 3	32 ± 2	35 ± 8	9
4	30 ± 2	39 ± 3	8
5	28 ± 3	38 ± 2	6
6	36 ± 2	28 ± 2	6
7	31 ± 2	32 ± 5	8
9	35 ± 2	29 ± 3	6
10	29 ± 2	34 ± 4	6
12	37 ± 3	40 ± 6	6
13	37 ± 3	27 ± 1	6
14	27 ± 3	25 ± 3	5
15	43 ± 2	26 ± 1	5
16	45 ± 4	27 ± 2	6

N.P. = Non-proliferative

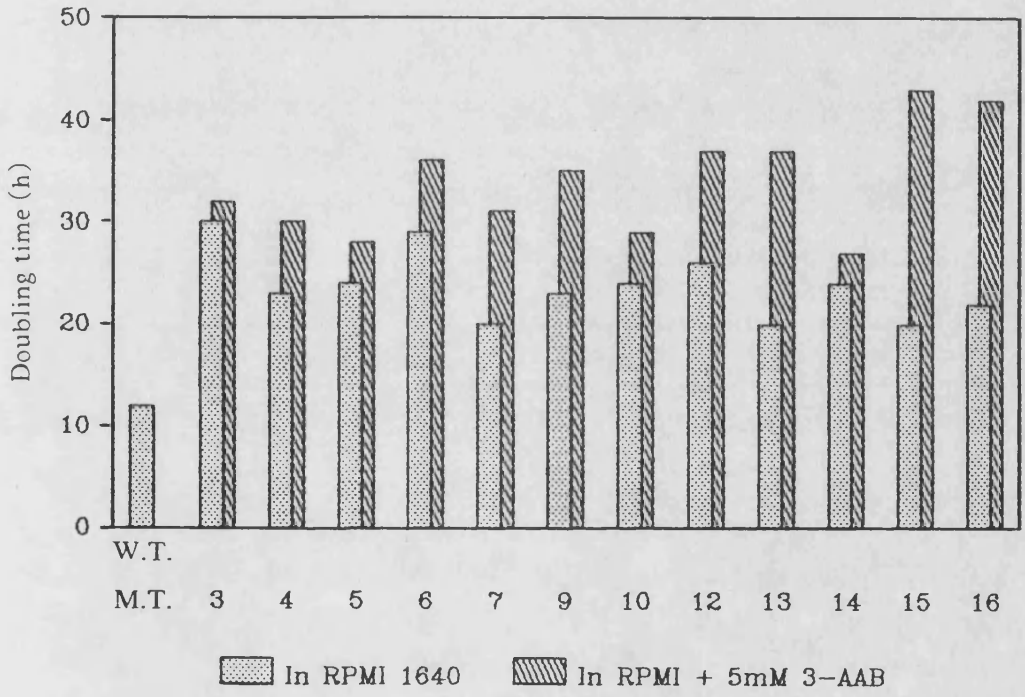
Cells (at $5x10^4$cells/ml) were grown in RPMI 1640 medium containing 5 mM 3-AAB over period of 96 h. At time intervals of 12 h the cell density was determined. Cell cycle doubling times were determined from slopes of growth curves, calculated from least square regression analysis of data.

TABLE 7 (A) Ratios of doubling time of wild-type (W.T.) to mutant cells (M.T.) [W.T. : M.T.]

(B) Ratios of doubling time of each mutant cell line in the absense of 5 mM 3-acetamidobenzamide (-AAB) to its doubling time in the presence of 5 mM 3-acetamidobenzamide (+AAB) [M.T. (-AAB) : M.T. (+AAB)]

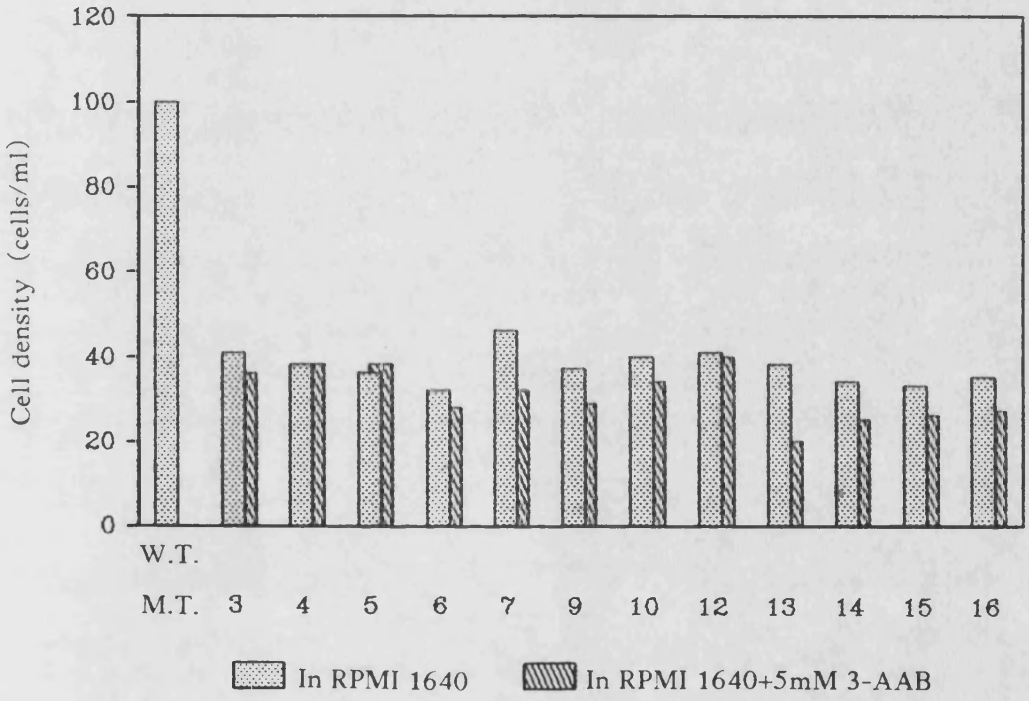
Mutant number	(A)	(B)
3	1 : 2.4	1 : 1.10
4	1 : 1.9	1 : 1.30
5	1 : 2.0	1 : 1.17
6	1 : 2.4	1 : 1.24
7	1 : 1.7	1 : 1.55
9	1 : 1.9	1 : 1.52
10	1 : 2.0	1 : 1.21
12	1 : 2.2	1 : 1.42
13	1 : 1.7	1 : 1.85
14	1 : 2.0	1 : 1.13
15	1 : 1.7	1 : 2.15
16	1 : 1.8	1 : 2.05

FIGURE 6.a Comparison of the doubling time of wild-type and mutant cell lines in the presence and absence of 5 mM 3-AAB (see also table 6 a & b).



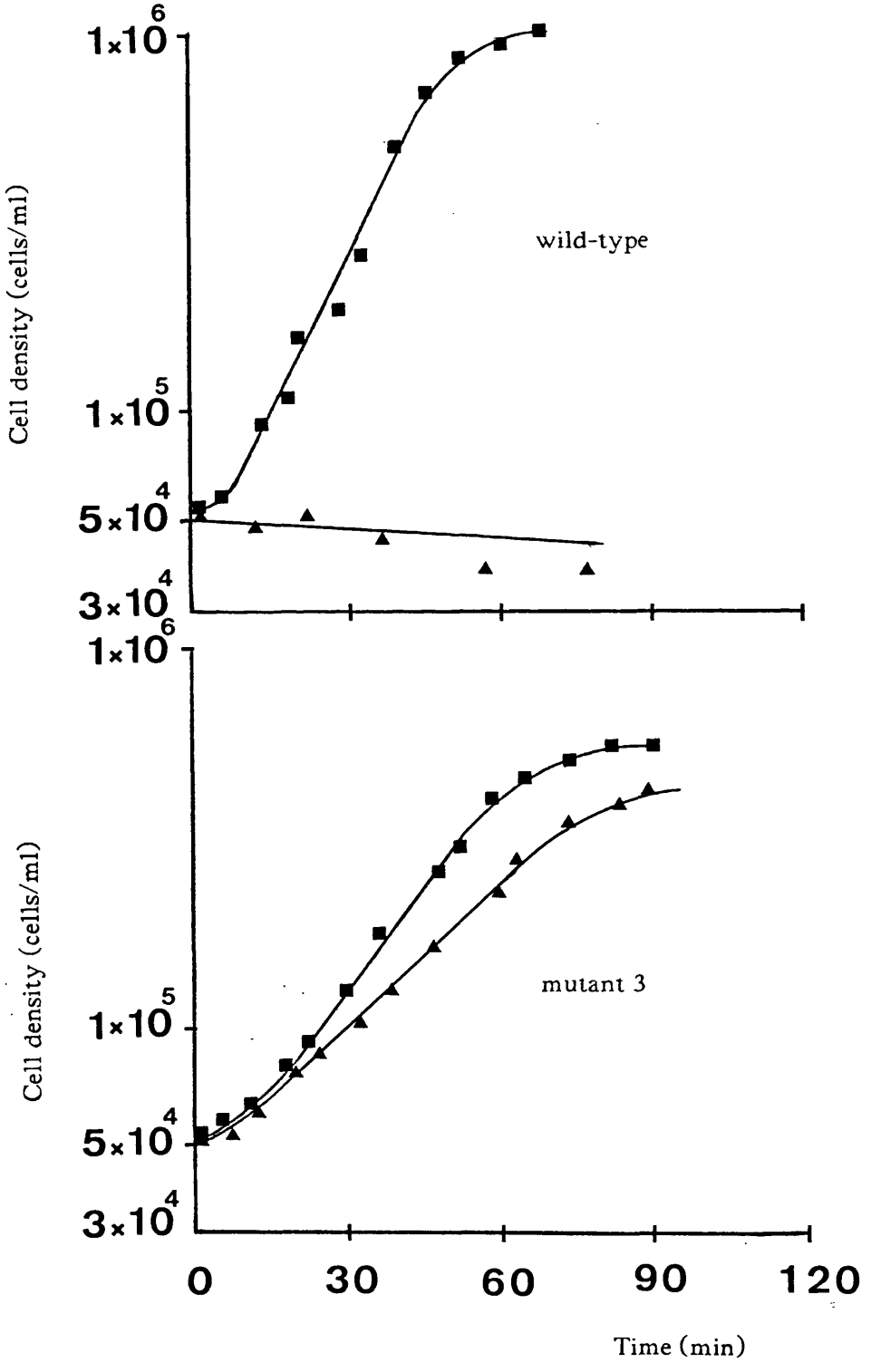
M.T.= Mutant W.T.= Wild-type

FIGURE 6.b Comparison of the maximum cell density of wild type and mutant cell lines at stationary phase in the presence and absence of 5 mM 3-AAB (see also table 6 a & b).



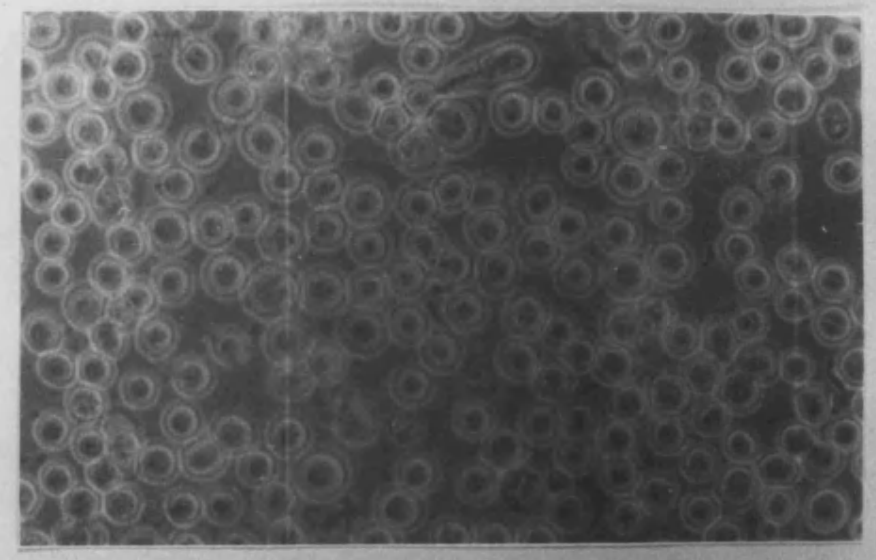
W.T.= Wild-type M.T.= Mutant

FIGURE 7 Growth curves of wild-type and mutant 3 cells in the presence and absence of 5mM 3-AAB (as described in table 6 a & b).

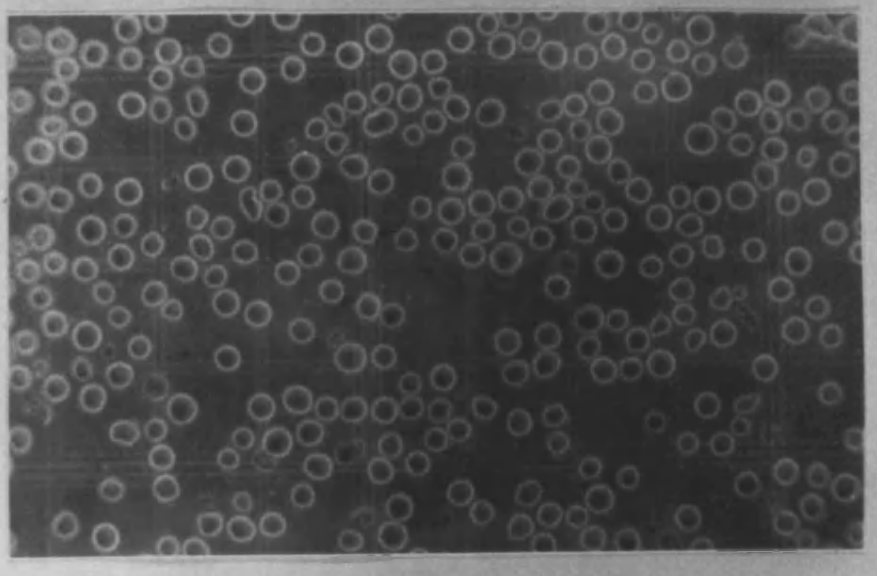


■ = in the absence of 3-AAB ; ▲=in the presence of 3-AAB

Figure 8 Picture of mutant 3 and wild-type cells



MUTANT 3 CELLS



WILD-TYPE CELLS

Diameter of mutant and wild-type cells using magiscan 2A

(from 100 cells): Diameter of mutant 3 cells = (1.611±1.8)x10 μm

Diameter of wild-type cells = (1.214±0.117)x10 μm

The ratio of diameter mutant: wild-type = 1:1.32

have a shorter doubling time of 12-13 h but they do not proliferate in 5 mM 3-AAB. It is evident that mutant cell lines have longer doubling times which are in the range of 20- 30 h and the maximum cell density of mutant cell lines are 2-3 fold less than wild-type cell density at the stationary phase (see figure 6a, b). The sizes of mutant cells are larger than wild-type cells (figure 8), and when viewed subjectively by the naked eye they were estimated to be one and a half times larger. As all the mutant cell lines have been shown (from microscope examination) to be of similar size, only the size of mutant 3 and wild-type cell lines were measured using a magiscan 2A machine (Joyce Loebl). This machine magnifies the sizes of cells and projects them on a screen which automatically reads off the diameter scale and prints it on the screen. The mean value of cell diameter of mutant 3 (from 100 cells sample) is 1.32 times larger than the average diameter of wild-type cells (see figure 8). The difference in cell density at the early stionary phase of wild-type and mutant cell lines may be due to the depletion of chemical substances in the medium, as bigger cells would have taken up more substances from the medium in order to synthesize various cellular components e.g. additional molecules of protein, nucleic acid, carbohydrates, lipids and other cellular constituents before cell division.

As seen from table 7 and figure 6(a), all mutant cell lines have a longer doubling time in the order of 1.6-2.3 times longer compared to wild-type cells. Results also show that the doubling times of mutant cell lines under selective pressure are longer than the doubling times obtained in the absence of selective pressure (figure 6.a). For mutants 13, 15 and 16, the doubling time is twice as long and for mutants 7, 9 and 12 the increase factor is about 1.5. The doubling time of other mutants is about 1.1-1.3 longer in the presence of 5 mM 3-AAB. It is interesting that mutant 3 has almost the same doubling time in the presence and absence of 5 mM 3-AAB. This shows that the inhibitor has very little effect on cell growth in this mutant.

The characteristics of the doubling times of mutantcell lines isolated with 5 mM 3-AAB are different compared to mutant cell lines obtained by other workers. Mutants of HeLa cells, selected in the presence of 10 mM 5-methylnicotinamide (Kidwell and Burdette 1974) and of L1210 cells isolated in the presence of 6 mM 5-methylnicotinamide (Nduka and Shall, 1980) have been shown to grow with similar doubling time to their wild-type in the absence of their selective pressure. The doubling time of 24 and 12 h were observed for the mutant and its wild-type of HeLa cells and for the mutant and its wild-type of L1210 cells respectively. The same result was also observed in variant L1210 isolated in the presence of 3-aminobenzamide after the cells were mutagenised by DMS (Murray *et al.*, 1986). Moreover, variant cells L25A isolated in the gradually increasing concentration of 3-aminobenzamide have a doubling time of 15 h compared to 12 h for its wild-type.

The difference in the length of cell cycle of mutant cell lines in the presence and absence of 5 mM 3-AAB (see figure 6.a) may be due to one of the cell cycle phases which contribute to the difference of cell cycle length observed. There is a generally accepted view that the variation of doubling time among different cell types is mainly due to the length of G_1 (Killander and Zetterberg, 1965; Worthington *et al.*, 1976), although in certain systems, e.g. mutant of *Tetrahymena thermophila* (Schafer and Cleffmann, 1982), it has been shown that the variation in cell cycle times of a mutant and its wild-type is due to G_1, S and G_2.

3-Acetamidobenzamide (3-AAB) may affect G_2 phase in cell cycle of mutant cell lines. This effect is concluded from reports obtained from using other inhibitors. For example variant L25A cells which are resistant to 25 mM 3-aminobenzamide were shown to have a slightly higher fraction of G_2 cells. (Tavassoli *et al.*, 1987a). 3-Methoxybenzamide was found to cause a large accumulation in G_2 such that the

first completion of mitosis was at 38 h instead of 26-28 h in untreated C3H10 T1/2 cells (Jacobson *et al.*, 1985a). Another report shows that using 5 mM 5-methylnicotinamide affects the G_2 of mutants of HeLa cells (resistant to 5mM 5-methylnicotinamide) (Kidwell and Burdette, 1974). 3-AAB may also affect G_1 phase as well as G_2. This conclusion is based on the report that 3-AAB effects growth arrest in G_1 and G_2 of HeLa cells (Kidwell *et al.*, 1985).

In response to selective pressure, mutant cell lines may enter into sub optimal growth conditions by entering the Go state as it was shown that various drugs cause cells to enter Go state (Pardee and James, 1975). Furthermore, a certain percentage of mutant cells in various cell lines may not undergo the process of cell division; instead, they may remain in Go state and this eventually results in cell death. To verify these observations, the sensitivity of mutant cell lines to various doses of 3-AAB was investigated in the next section.

4.2.2 Cytotoxicity of 3-acetamidobenzamide (3-AAB) to mutant cell lines

Cell survival in soft agar is used to measure the effect of 3- AAB on mutant cell lines. For each treatment, a group of 100 cells for each mutant cell line was plated on to a petri dish with no inhibitor (control), 1, 3, 4, 5, and 6 mM 3-AAB and 500 cells for 7 mM 3-AAB. Five petri dishes were prepared for each independent experiment. After 4 weeks of incubation at 37 deg.C and 5% CO_2, inside the plastic box (as described in 4.1.4), colonies were counted from each treatment group and calculated as percentage survival from the equation below.

$$\frac{\text{No of colonies in the presence of 3 AAB (+)}}{\text{No of colonies in the absence of 3 AAB (-)}} \times 100\ \%$$

The plating efficiency of mutant and wild-type cell lines which had received no 3-AAB is in the range of 40-70% (30 determinations for each mutant cell line) and 72±4.9% for wild-type cells (50 determinations). The percentage survival of mutant cell lines and wild-type cells after treatment with various concentrations of 3-AAB is plotted as a semi log plot in order that a direct comparison be made [see figure 9 (a-d)]. Table 8 shows the percentage survival of mutant cell lines in the presence of 5 mM 3-AAB and table 9 shows the concentration of 3-AAB which allows 10% of mutant and wild-type cell lines to survive.

DISCUSSION

The percentage survival of mutant cell lines obtained in 5 mM 3-AAB is shown in table 8. The mean value of percentage survival obtained from 3 independent experiments (5 petri dishes per each independent experiment) for most mutant cell lines, in 5 mM 3-AAB, is above 50% except mutant 16 which is 35%. A 50% survival for wild-type was achieved at 1.5 mM (figure 9.a) showing that a 3 fold increase in 3-AAB concentration for mutant cell lines was needed to achieve a similar effect. Concentrations of 3-AAB [extracted from figure 9 (a-d)] which allowed 10% cell survival in wild-type and mutant cell lines and their ratio are summarized in table 9 and presented as graph in figure 10. Results show that the concentration of 3-AAB which allows 10% of mutant cells to survive is increased by a factor of 2.5-3.0 (compared to wild-type), with a slightly higher factor of 3.4 for mutant 13.

Results from table 8 and 9 clearly demonstrate that mutant cell lines are more resistant to the cytotoxicity of 3-AAB. Higher doses were needed for mutant cell

TABLE 8 Percentage (%) survival of mutant cell lines treated continuously with 5 mM 3-AAB in agar plating. Each data point was calculated from 5 separate experiments.

Cell lines	1st expt	2nd expt	3rd expt	Mean value
	%	%	%	
Mutant 3	41 ± 10	78 ± 9	35 ± 8	51
4	52 ± 10	80 ± 10	42 ± 6	57
5	61 ± 4	80 ± 6	45 ± 7	62
6	38 ± 4	55 ± 7	80 ± 8	57
7	62 ± 7	30 ± 7	62 ± 9	52
9	49 ± 7	52 ± 7	83 ± 10	61
10	59 ± 7	45 ± 6	90 ± 8	65
12	53 ± 5	45 ± 5	82 ± 7	60
13	70 ± 10	79 ± 8	60 ± 5	69
14	69 ± 9	47 ± 9	87 ± 7	67
15	40 ± 8	52 ± 8	75 ± 10	55
16	28 ± 3	44 ± 4	35 ± 4	35

Mutant cells of various cell lines were diluted and plated (100 cells) onto 6 cm petri dish in media containing 5 mM 3- AAB. After 4 weeks, colonies were counted and calculated as percentage survival according to the equation below:

$$\frac{\text{No of colonies in the presence of 5 mM 3-AAB x 100\%}}{\text{No of colonies in the absence of 5 mM 3-AAB}}$$

TABLE 9 (A) = Various concentrations (mM) of 3 acetamidobenzamide (3-AAB) which allow 10% of mutant and wild-type cell lines to survive in the continuous treatment of 3-AAB in agar plating (extracted from figure 9 a, b, c, d).

(B) = Ratio of 3-AAB (mM) concentrations (shown in colum A) which allow 10% survival of mutant cells to wild-type cells.

As a:b

a) 3-AAB (mM) giving 10% survival of mutant cells

b) 3-AAB (mM) giving 10% survival of wild-type cells

Cell lines	(A)	(B)
Wild-type	2.5	-
Mutant 3	6.7	2.7 : 1
4	6.5	2.6 : 1
5	7.5	3.0 : 1
6	7.5	3.0 : 1
7	7.0	2.8 : 1
9	7.5	3.0 : 1
10	7.5	3.0 : 1
12	7.5	3.0 : 1
13	8.5	3.4 : 1
14	6.5	2.6 : 1
15	6.25	2.5 : 1
16	6.25	2.5 : 1

FIGURE 9.a Cytotoxicity of 3-AAB on wild-type and mutants 3,4,5 cell lines treated continuously in soft agar (see "Methods" chapter 3.4.5). (5 experiments per each data point).

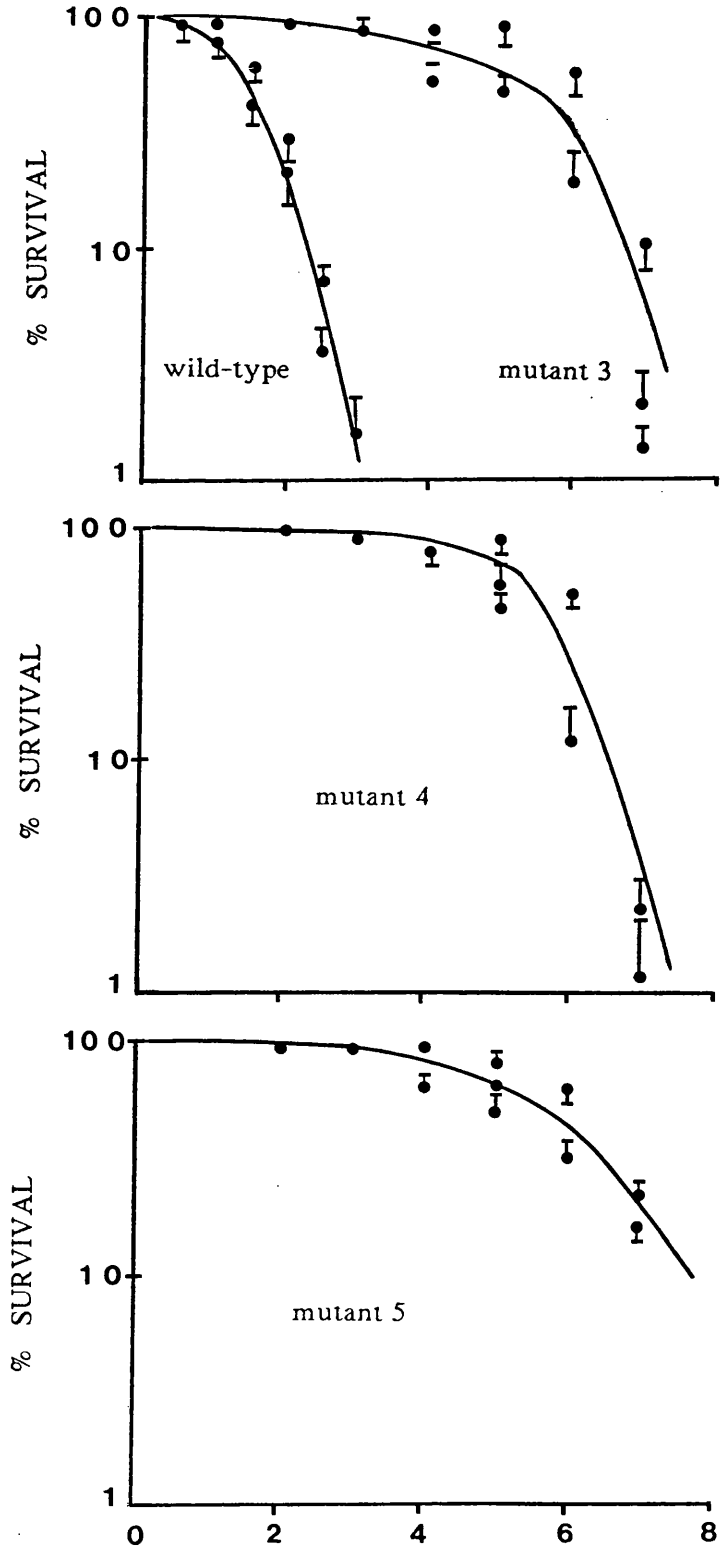


FIGURE 9.b Cytotoxicity of 3-AAB on mutants 6,7,9 cell lines treated continuously in soft agar (see "Methods" chapter 3.4.5). (5 experiments per each data point).

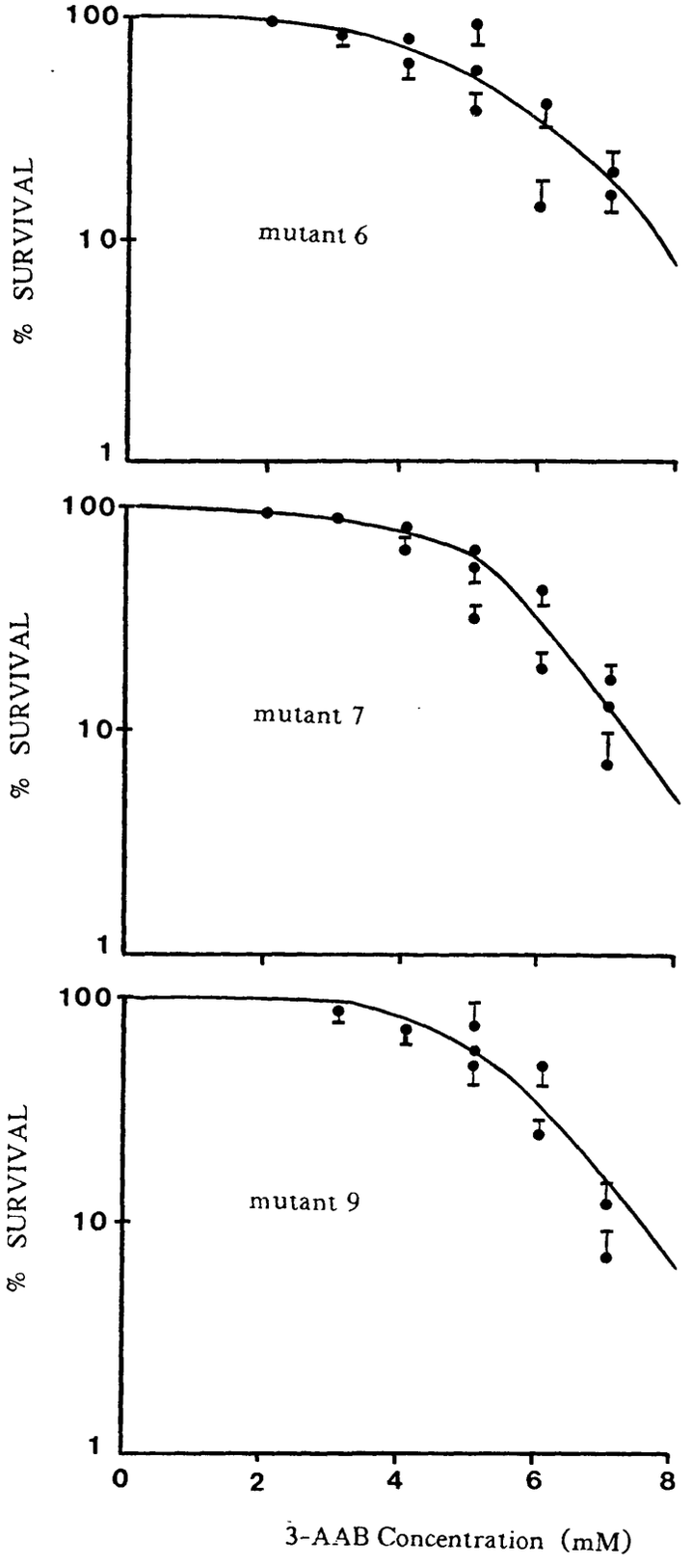


FIGURE 9.c Cytotoxicity of 3-AAB on mutants 10,12,13 cell lines treated continuously in soft agar (see "Methods" chapter 3.4.5). (5 experiments per each data point)

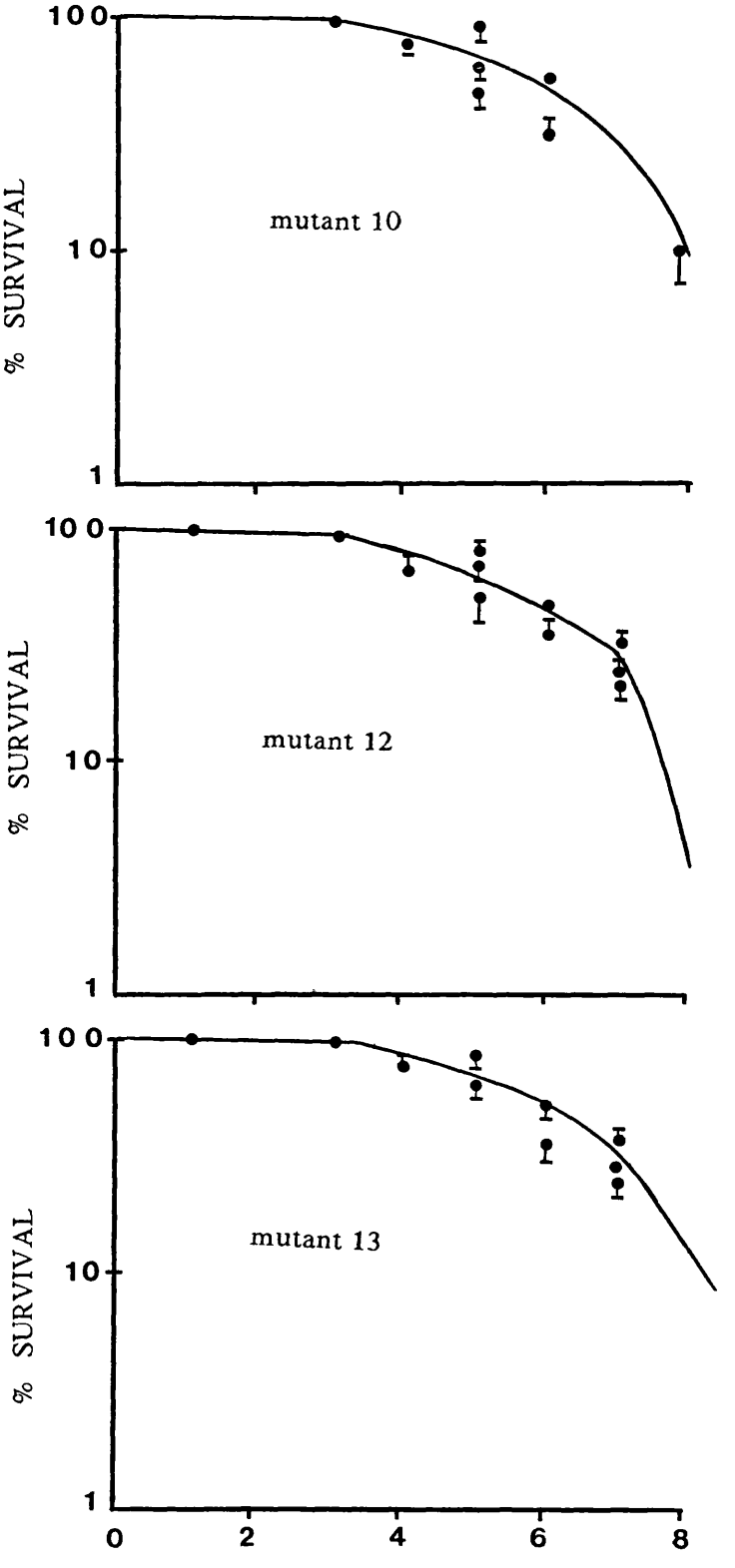


FIGURE 9.d Cytotoxicity of 3-AAB on mutants 14,15,16 cell lines treated continuously in soft agar (see "Methods" chapter 3.4.5). (5 experiments per each data point).

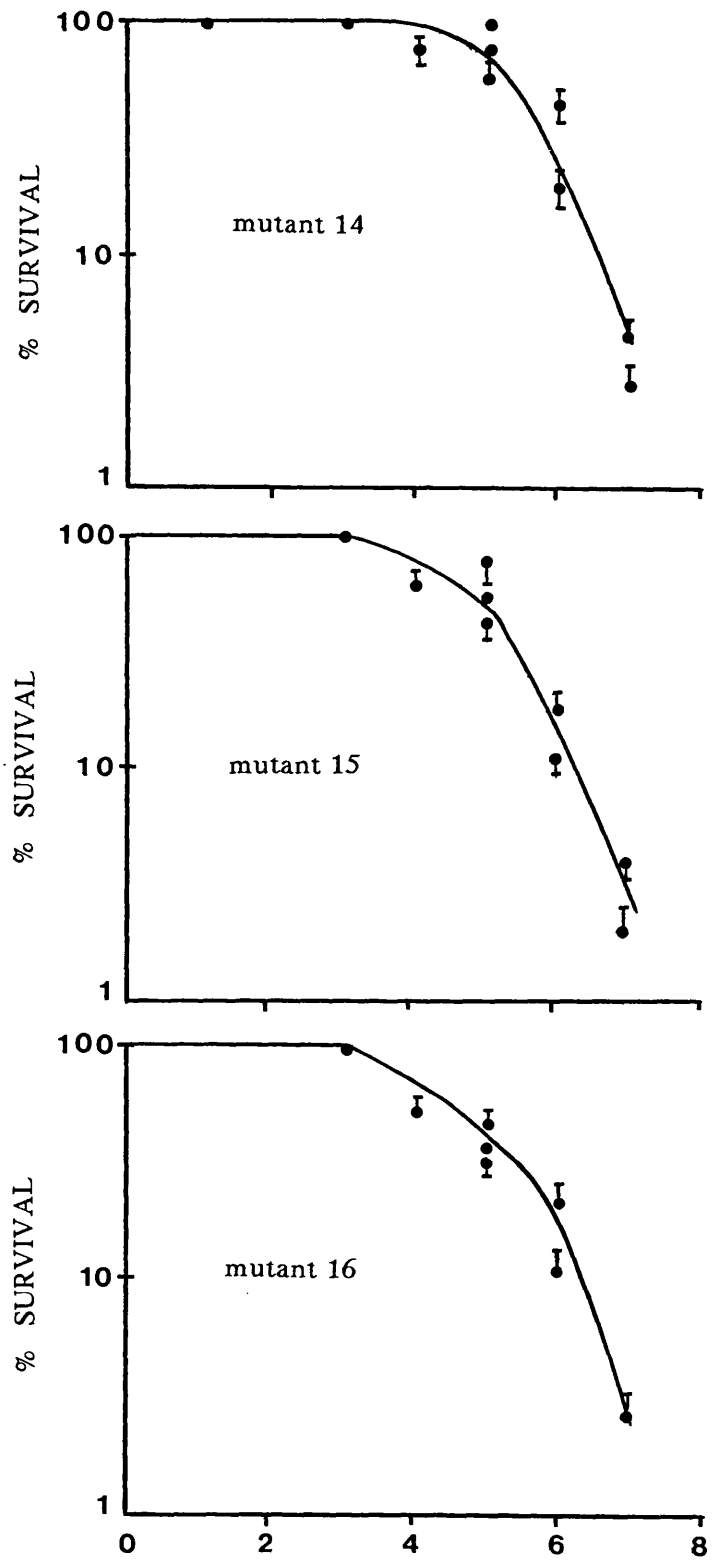
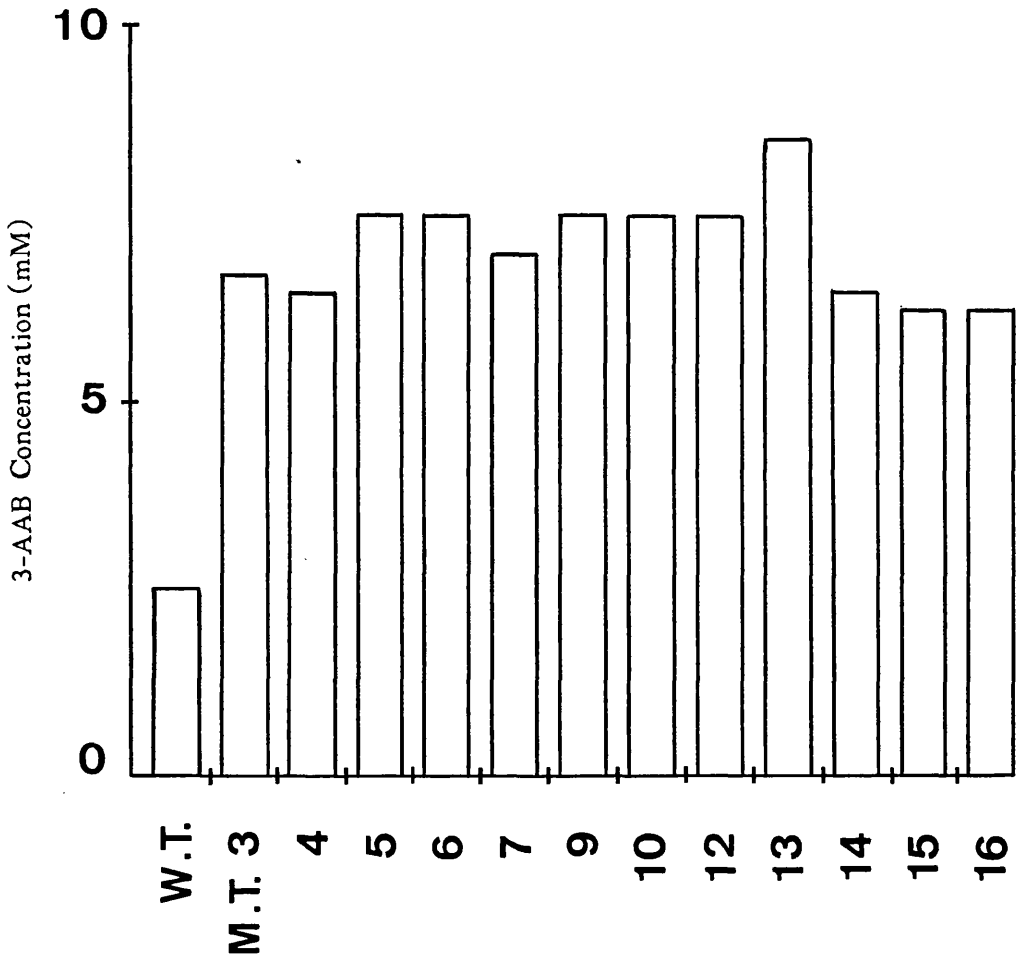


FIGURE 10 Different concentrations of 3-AAB which allow 10% of wild-type and various mutant cells lines to survive (see also table 9 (A)).



W.T.= Wild-type M.T.=Mutant

lines in order to obtain the same effect as that found for wild-type cells. In wild-type cells, the cytotoxicity of 3-AAB increased dramatically at concentrations above those giving 70% survival, whereas in mutant cell lines it was achieved at concentrations above those giving 30% survival. However, as seen on survival curves [figure 9 (a-d)], the increase in cytotoxicity of 3-AAB on mutant cell lines was more gradual compared to the survival curve of wild-type cells.

Results obtained so far indicate that mutant cell lines are defective in certain functions related to the inhibitory effect of 3-AAB which normally affects wild-type cells.

4.2.3 Cell membrane permeability to 3-AAB

The transport of 3-AAB across the cell membrane has been investigated to ascertain whether mutation is affecting cell membrane permeability. Mutant cells grown in suspension medium of RPMI 1640 (no inhibitor) were collected (during mid- log phase, at 2.0-2.5x10^5 cells/ml) by aspirating cells to give a final cell density of 5x10^6 cells/ml. A total volume of 3 ml was incubated with 15 µl of [^{3}H]3-AAB. At various time intervals of 5 min, 30 min, 1, 2 and 3 h; 1x10^6 cells (200 µl) were filtered through a Whatman GFC disc and washed with 3x10 ml of ice cold PBS.

For the background control, untreated cells (1x10^6 cells) filtered through a GFC disc were washed with an equal volume (200 µl) of radioactive medium followed by the washing procedure of PBS. The counts obtained provided the background of radioactive counts which were not inside the cells.

The discs were dried at room temperature and the radioactivity determined (see method chapter 3.7). The results are presented in table 10 and figure 11 and they show that 3-AAB diffused into the cells to reach equilibrium very quickly as the radioactive count (cpm) at 5 min and 3 h are very similar. Results show that the amount of 3-AAB accumulated in various mutant cell lines is greater compared to wild-type cells (as shown by the radioactive count per 10^6 cells): it is increased by a factor in the range of 2.0-2.8. This increased factor is probably due to the sizes of mutant cells which are larger (see figure 8) than the wild-type, hence mutant cells accumulate more 3- AAB inside themselves. This experiment demonstrates that mutation in mutant cells was not due to their cell membrane permeability as 3-AAB passes through the membranes.

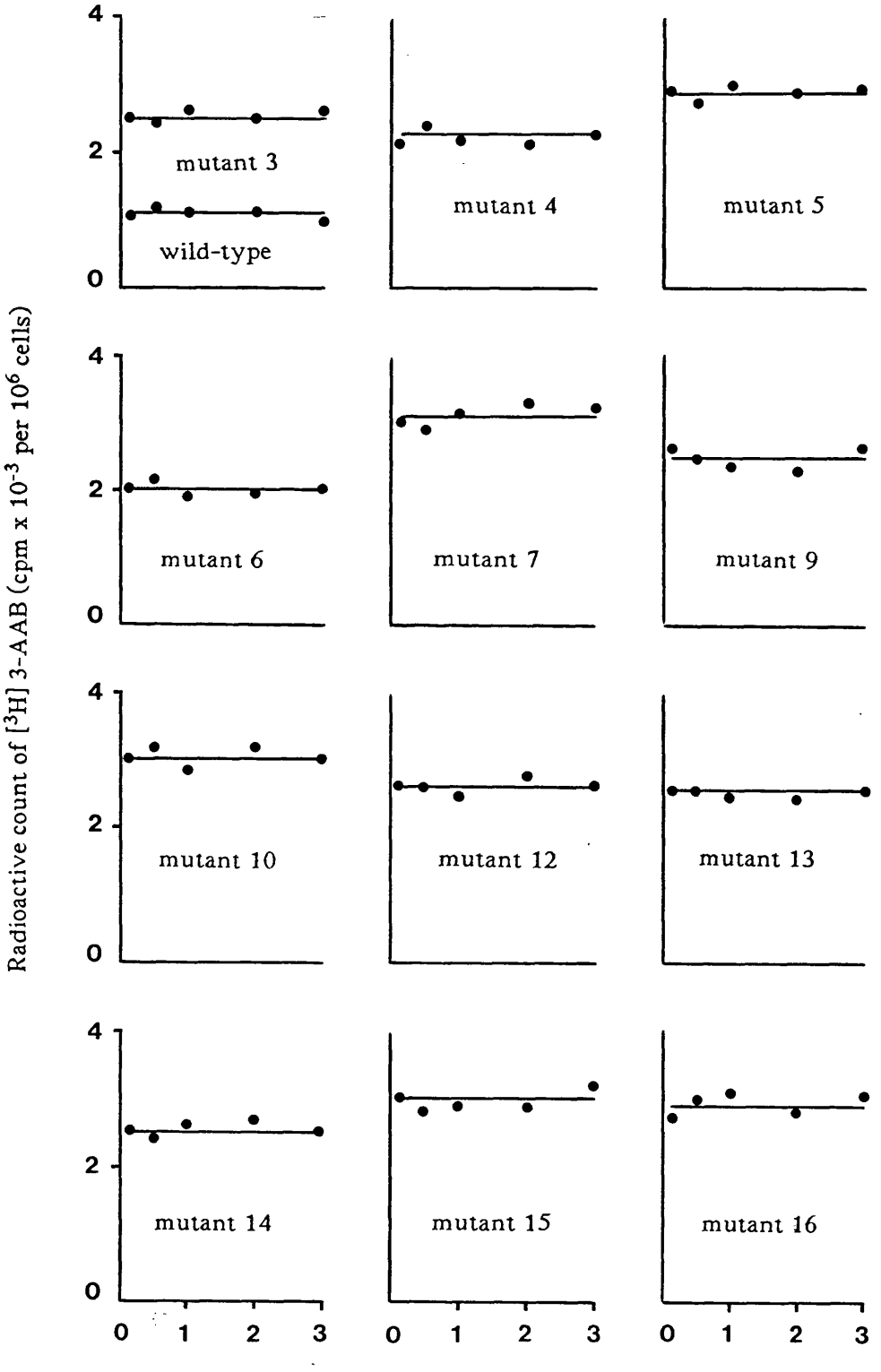
TABLE 10 Radioactive count of [^{3}H] 3-acetamidobenzamide, presented as c.p.m. x 10^{3} per 10^{6} cells in wild-type and various mutant cell lines. Each data point consists of an average of 3 determinations (see also figure 11).

Cell lines	Incubation Times						
	5 min	30 min	1 h	2 h	3 h	Mean value	I.F.
Wild-type	1.1±.07	1.2±.06	1.1±.03	1.1±.06	1.0±.09	1.1±.05	1
Mutant 3	2.5±.07	2.4±.09	2.6±.12	2.5±.08	2.6±.12	2.5±.08	2.3
4	2.1±.05	2.4±.11	2.2±.12	2.1±.07	2.3±.07	2.2±.13	2.0
5	2.9±.12	2.8±.12	3.0±.15	2.9±.16	2.9±.15	2.9±.07	2.6
6	2.1±.02	2.2±.10	2.0±.16	2.0±.07	2.0±.12	2.0±.08	1.8
7	3.0±.14	2.9±.16	3.1±.18	3.3±.10	3.2±.19	3.1±.15	2.8
9	2.7±.06	2.5±.12	2.4±.12	2.3±.06	2.6±.10	2.5±.15	2.3
10	3.0±.12	3.2±.11	2.9±.15	3.2±.05	3.0±.12	3.0±.13	2.7
12	2.6±.15	2.6±.17	2.5±.12	2.7±.11	2.6±.09	2.6±.07	2.4
13	2.6±.12	2.6±.17	2.5±.15	2.4±.15	2.5±.18	2.5±.08	2.3
14	2.6±.09	2.5±.06	2.6±.16	2.8±.08	2.5±.09	2.6±.08	2.4
15	3.0±.17	2.8±.12	2.9±.15	2.9±.15	3.2±.20	3.0±.15	2.7
16	2.8±.15	3.0±.13	3.1±.17	2.9±.15	3.2±.15	3.0±.16	2.7

I.F. = the ratio of uptake of [^{3}H] 3-AAB in wild-type to each mutant cell line.

Wild-type and mutant cell lines ($5x10^{6}$ cells/ml; total volume 3ml) were incubated with 15 µl of [^{3}H] 3-AAB (117 nCi/ml). At various time intervals, $1x10^{6}$ cells were filtered through a glass fibre disc and washed with 3x10 ml PBS. The radioactivity of [^{3}H] 3-AAB inside the cells was determined according to the methods in chapter 3.3.

FIGURE 11 Radioactivity count of [^{3}H] 3-AAB transported inside wild-type and mutant cell at various incubation periods (see also table 10).



CHAPTER 5

THE CHARACTERIZATION OF ADP-RIBOSYLATION IN VARIOUS MUTANT CELL LINES

CHAPTER 5

Mutant cell lines were tested in the following sections to determine whether or not they contain the enzyme ADPR transferase and whether their NAD biosynthesis is maintained at the normal levels using [^{14}C] nicotinamide as a precursor. Mutant cell lines were also tested for their responses to DNA damaging agents in the presence and absence of 3- acetamidobenzamide(3-AAB), as ADP-ribosylation is known to be involved in DNA repair (chapter 1.1.9). The results obtained would provide the preliminary evidence if their survival in high concentration of 3-AAB (a concentration toxic to wild- type cells) is related to ADP-ribosylation. Furthermore, the cytotoxicity of 3-aminobenzamide and 3-nitrobenzamide (also ADPR transferase inhibitors) together with their acid analogues were tested on all mutant cell lines to determine if they are also resistant to high concentrations of these compounds.

5.1 AN INVESTIGATION OF ADPR TRANSFERASE ACTIVITY IN PERMEABILIZED MUTANT AND WILD-TYPE CELL LINES

ADPR transferase is a nuclear enzyme which catalyses the synthesis of poly (ADP-ribose) (chapter 1.1.3). The enzyme cleaves NAD to yield free nicotinamide and ADP-ribose moiety. It also transfers ADP-ribose to various acceptor proteins such as histones, RNA polymerase or even to ADPR transferase itself (chapter 1.1.4).

The substrate for ADPR transferase is NAD, a macromolecule which can not be supplied directly to the interior of intact cells. Most investigations of poly (ADP-ribose) synthesis in mammalian cell lines have therefore been conducted by permeable cell techniques, in which cells could be rendered permeable to exogenously supplied nucleotide. The method of cell permeabilization used for this investigation is based on the method of Durkacz *et al* (1980 a), which has been adopted from the original development of Berger *et al* (1978). They have demonstrated that studies in the permeable cells provide a more physiological assessment of the poly (ADP-ribose) synthesis than could be obtained by studies with isolated nuclei.

For the experimental system in this section, wild-type and mutant cell lines were prepared (see chapter 3.4.4) and used during log growth phase; 5-7x10^5cells/ml for wild-type cells and 2-2.5x10^5cells/ml for mutant cells (as the maximum cell density during early stionary phase is 10^6cells/ml for wild- type and 3.5-5 x10^5cells/ml for various mutant cell lines). Cells were pelleted in a bench centrifuge and resuspended in hypotonic buffer (as described in chapter 3.10) at 4 deg.C. Aliquots of mutant cells were taken at various times and mixed with an

equal volume of trypan blue and observed under the microscope. Microscopic view of a population of permeabilized mutant cells showed a mixture of both permeable and non- permeabilized cells such that only 30% of mutant cells were permeabilized after 15 min incubation period. A maximum of 75% of the cells were permeabilized after 45-50 min, leaving the other 25% unpermeabilized even if the incubation period was extended. The suspension of mutant cells in hypotonic buffer was, therefore, left on ice for 45-50 min before being diluted 10 fold with isotonic buffer (see chapter 3.10) ready for the assay.

However, 95-100% of wild-type cells were permeabilized within 15-20 min, but as the enzyme activity in mutant cell lines was to be compared with the wild-type, all cell lines were therefore treated under the same experimental conditions (ie wild-type cells were incubated with hypotonic buffer for 50 min). The ADPR transferase activity of wild-type cells (measured as initial rate of reaction) determined after 15 min was shown to be within the maximum upper limit of standard deviation of enzyme activity determined after 50 min incubation. (see figure 13a). For each experiment, 5 µl of [^{3}H] NAD in 50% ethanol (v/v) (23 mCi/µmole) was added to 245 µl of cells in buffer solution ($0.5x10^6$cells). The experiment was carried out at 26 deg.C and a 25 µl sample was taken at various time points up to 5 min. The ADPR transferase was determined by measuring the incorporation of [^{3}H] NAD into acid-insoluble material as described in chapter 3 (sections 7 and 8)

When ADPR transferase was assayed in the presence of 1mM 3-acetamidobenzamide, the inhibitor was added 5 min prior to the ADPR transferase assay.

RESULTS

The ADPR transferase activity in wild-type and mutant cell lines was measured for 5 min. Figure 12 (a-d) shows a typical time course of poly (ADP-ribose) synthesis in various cell lines measured as the incorporation of radioactive ADP-ribose from [^{3}H] NAD into TCA products in the presence and absence of 1mM 3- acetamidobenzamide. A time course of poly (ADP-ribose) synthesis obtained for wild-type cells showed an initial rapid uptake for the first 1.5-2 min which was linear followed by a much slower rate of synthesis (non-linear) up to the incubation time of 5 min (figure 12a). A similar time-course was also observed in mutants 6, 9, 13 and 16 (figure 12b), although a small decrease in poly (ADP-ribose) synthesis in these cells after 3-5 min was observed. This small decrease (or decay) was not so significant compared to the rapid decreases obtained in other mutant cell lines shown in figure 12 (c&d).

A time-course of poly (ADP-ribose) synthesis in mutants 7 and 14 (figure 12c) showed a rapid rate of uptake in the first 1 min period followed by a decrease in the rate of uptake between 2-3 min for mutant 7, and for mutant 14 this remained level for 1 min. This was prior to a gradual decrease in the rate of polymer synthesis of 0.08±0.01 and 0.05±0.02 pmole/min/10^6cells respectively.

Poly (ADP-ribose) synthesis obtained for mutants 3, 4, 5, 10, 12, and 15 also showed rapid initial rates of reaction for the first 1-2 min (as obtained in wild-type cells). Instead of continuing to maintain their incorporation rates of poly (ADP-ribose) synthesis in the non-linear fashion during the plateau as observed in wild-type cells, however, the poly (ADP-ribose) synthesis showed a rapid decrease which was linear (figure 12 d)

Figure 12 (a-d) clearly showed that ADPR transferase activity in all mutant cell lines was completely inhibited by 1 mM 3-acetamidobenzamide.

The initial rate of poly (ADP-ribose) synthesis (pmole/min/10^6 cells) and the maximum amount of polymer synthesis of wild- type and mutant cell lines together with the rate of decay (pmole/min/10^6cells) observed in some mutant cell lines are summarized in table 11 and presented as graphs in figures 13 (a, b and c respectively).

The majority of mutant cell lines exhibited similar initial reaction rates of poly (ADP-ribose) synthesis to wild- type (figure 13 a) which were within the range of (0.41 - 0.67) p mole/min/10^6cells. The initial reaction rates of poly (ADP-ribose) synthesis in mutants 3 and 4 however were shown to be 50% lower compared to wild-type cells. The initial reaction rates of incorporation of wild-type cells, mutants 3 and 4 were 0.59, 0.25 and 0.31 pmole/min/10^6cells respectively.

Figure 13 (b) shows the maximum amount of poly (ADP-ribose) synthesized (plateau) in wild-type and mutant cell lines. The maximum amount of polymers obtained during plateau period in most mutant cell lines (except mutants 3 and 4) were within the same range of standard deviation of wild-type cells. There was a marked difference in the maximum amount of poly (ADP-ribose) synthesized in wild-type cells and mutants 3, 4 and 10. For mutants 3 and 4 it was in the order of 3 fold lower and in mutant 10, 2 fold lower.

After the plateau, the amount of NAD incorporated in some mutant cell lines decreased. This was observed as the rate of decay of poly (ADP-ribose) which was observed in mutants 3, 4 , 5, 7, 10, 12, 14 and 15 (mutants shown on figure 12 c,d).

TABLE 11 The summary of poly (ADP-ribose) synthesis in wild-type and mutant cell lines measured as the incorporation of [^{3}H] ADPR from [^{3}H] NAD in permeabilized cells (as described and presented in figure 12).

	A	B	C	
Cell line number	pmole/min/ 10^6 cells	pmole/ 10^6 cells	pmole/min/ 10^6 cells	No. of expts
Wild-type	.59±.18	1.03±.24	NONE	7
Mutant 3	.25±.05	.33+.09	.06±.02	8
4	.31±.03	.37±.10	.07±.01	3
5	.51±.12	.80±.13	.09±.03	4
6	.54±.15	.86±.17	N.S.	3
7	.55±.11	.80±.19	.08±.01	6
9	.62±.18	.72±.23	N.S.	3
10	.41±.06	.47±.10	.08±.03	3
12	.50±.08	.73±.15	.06±.03	3
13	.53±.19	.65±.21	N.S.	4
14	.65±.19	.78±.18	.05±.02	3
15	.67±.20	.80±.16	.09±.01	3
16	.52±.18	.86±.21	N.S.	4

N.S. = not significant

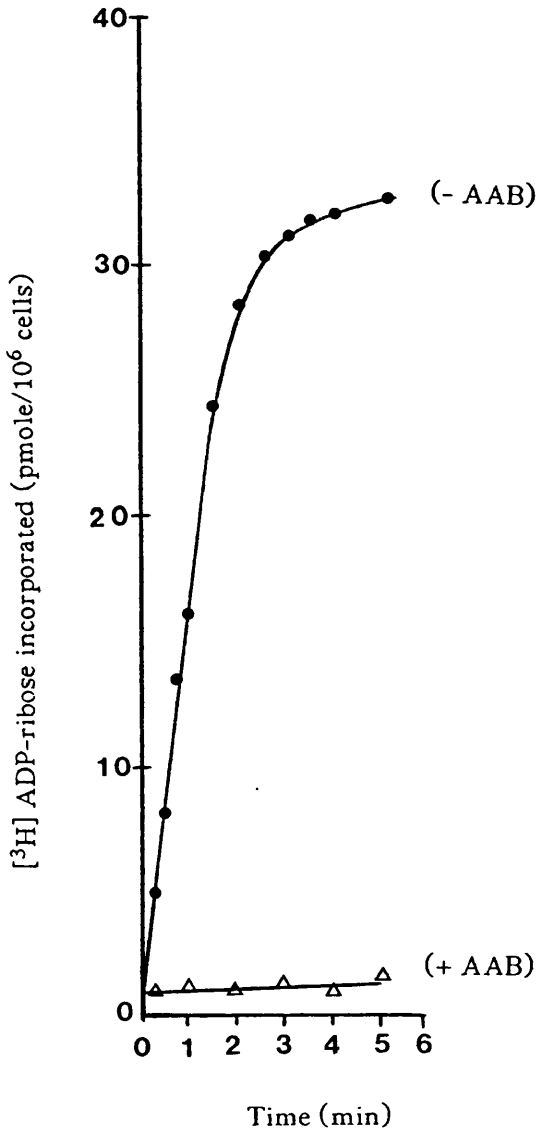
(A) The initial rate of poly (ADP-ribose) synthesis for wild- type and mutant cell lines ([^{3}H] ADPR incorporated , pmole/min/10^6 cells). (See also figure 13 a).

TABLE 11 (continue)

(B) The maximum amount of poly (ADP-ribose) synthesized during the plateau period (maximum [^{3}H] ADP-ribose incorporated (pmole/10^6 cells). (See also figure 13 b).

(C) The rate of decay of poly (ADP-ribose) after the plateau period (decay of [^{3}H] ADP-ribose (pmole/min/10^6 cells). (See also figure 13 c).

FIGURE 12.a Time course of poly (ADP-ribose) synthesis in wild-type cells measured as the incorporation of [^{3}H] ADPR from [^{3}H] NAD in permeabilized cells in the presence (+AAB) and absence (-AAB) of 1mM 3-AAB.(Graph shows a typical time course of several experiments).



Cells ($0.5x10^6$) were incubated with 5 µl [^{3}H] NAD in a total volume of 250 µl. At various time intervals, 25 µl was spotted on to Whatman filter paper disc (presoaked in 20% (w/v) TCA). The discs were washed and radioactivity determined according to methods described in chapter 3 (sections 7 and 8).

FIGURE 12.b Time course of Poly.(ADP-ribose) synthesis in mutants 6,9,13,16 cells measured as the incorporation of [^{3}H] ADPR from [^{3}H] NAD in permeabilized cells in the presence (+AAB) and absence (-AAB) of 1 mM 3-AAB, as described in figure 12.a.
(Graphs show a typical time course of several experiments).

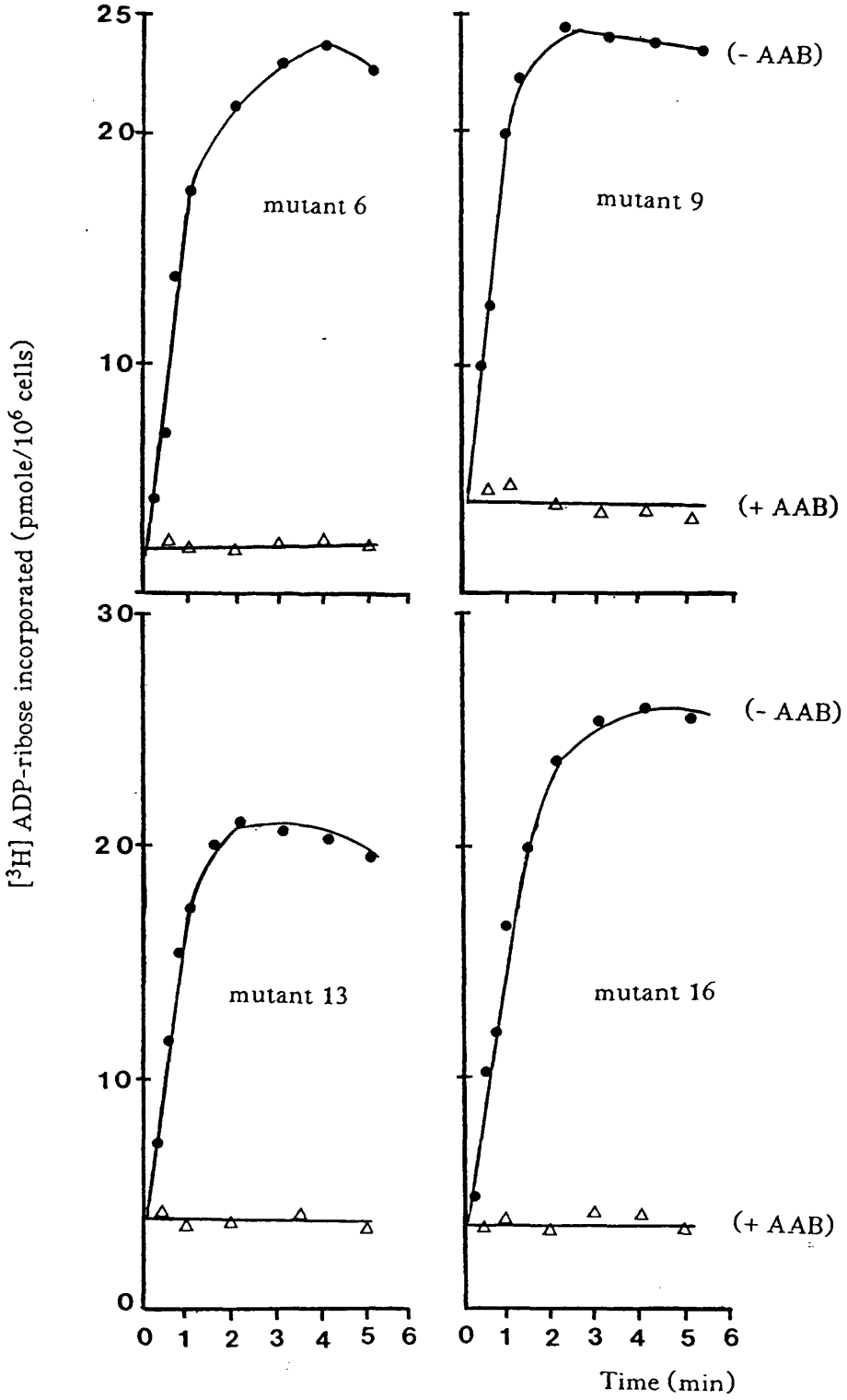


FIGURE 12.c Time course of poly (ADP-ribose) synthesis in mutants 7,14cells measured as the incorporation of [^{3}H] ADPR from [^{3}H] NAD in permeabilized cells in the presence (+AAB) and absence (-AAB) of 1 mM 3-AAB, as described in figure 12.a.

(Graphs show a typical time course of several experiments).

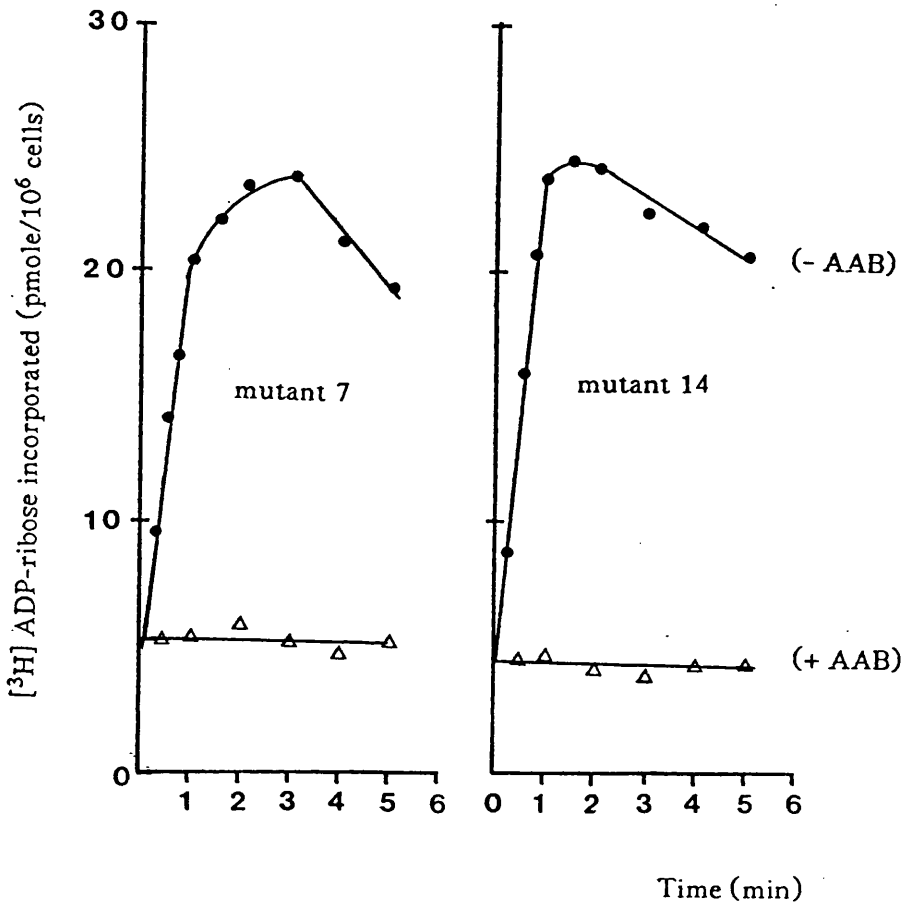


FIGURE 12.d Time course of Poly (ADP-ribose) synthesis in mutants 3,4,10,5,12,15 cells measured as the incorporation of [^{3}H] ADPR from [^{3}H] NAD in permeabilized cells in the presence (+AAB) and absence (-AAB) of 1 mM 3-AAB, as described in figure 12.a.

(Graphs show a typical time course of several experiments).

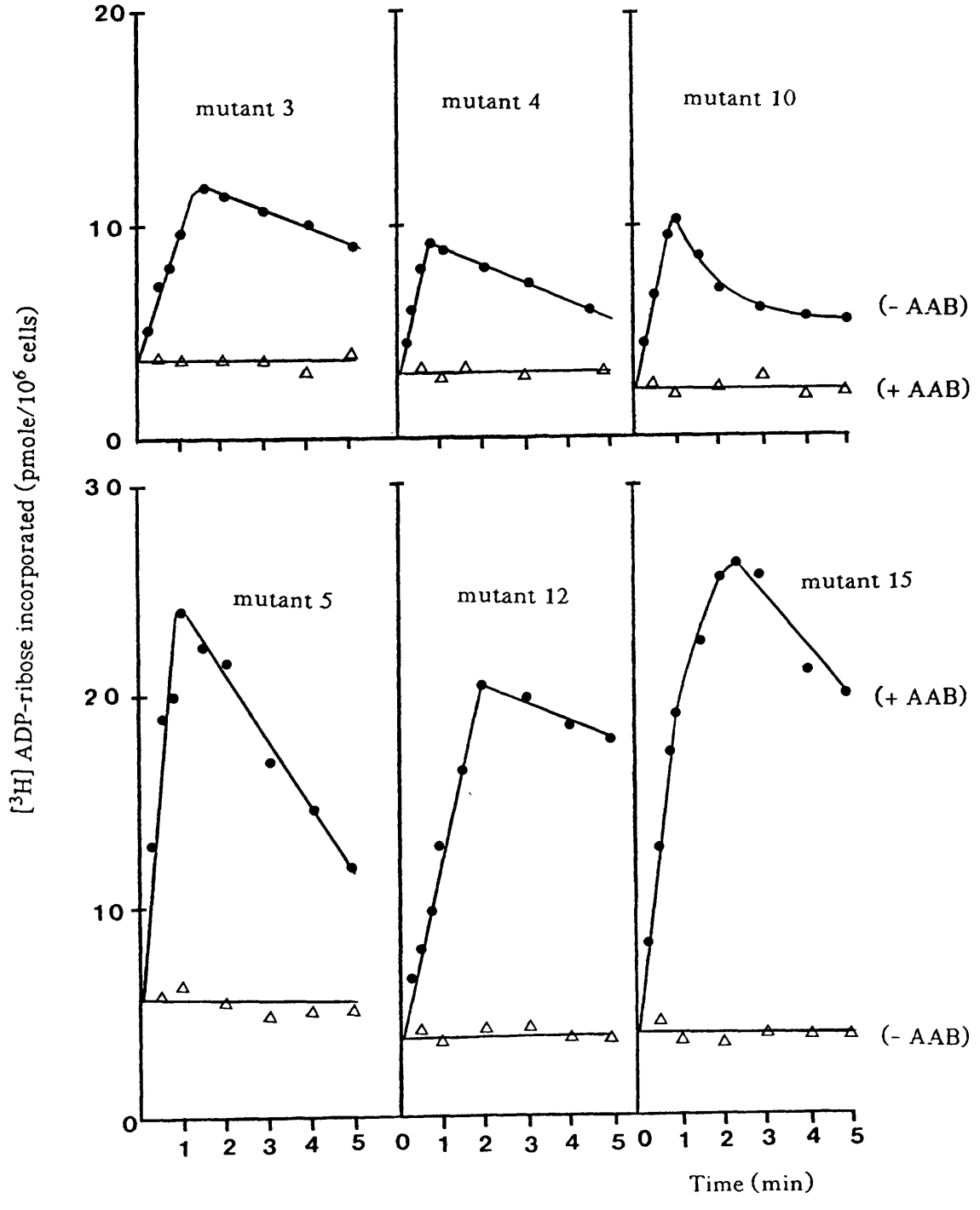
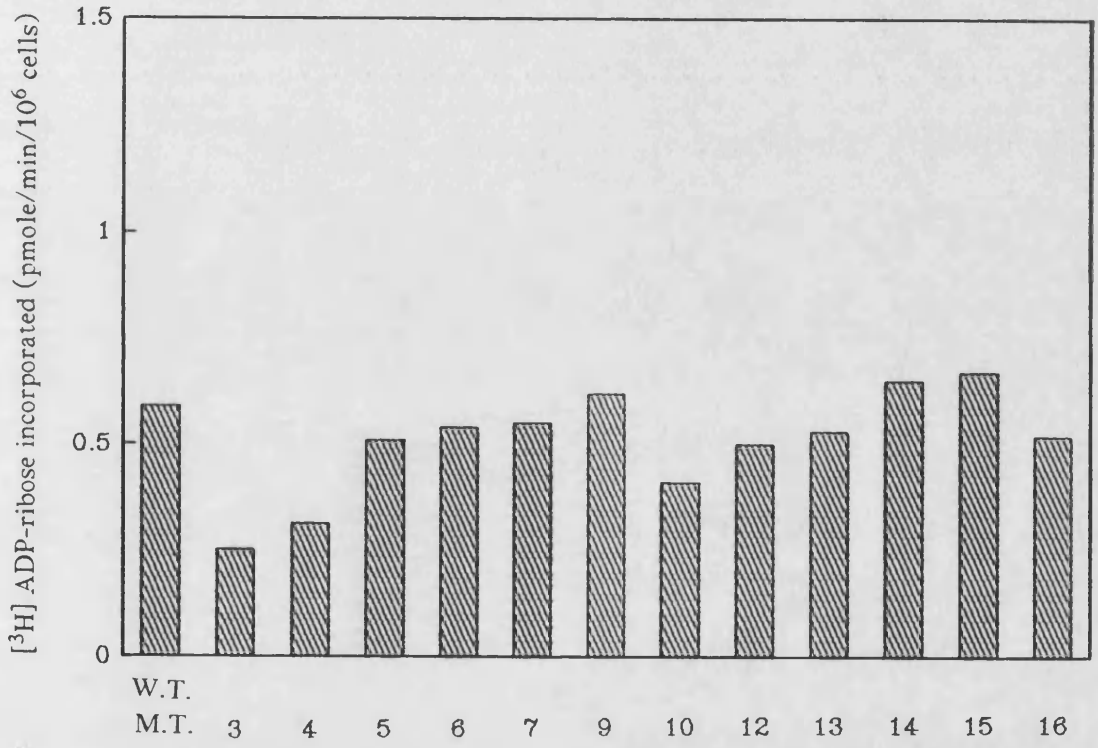
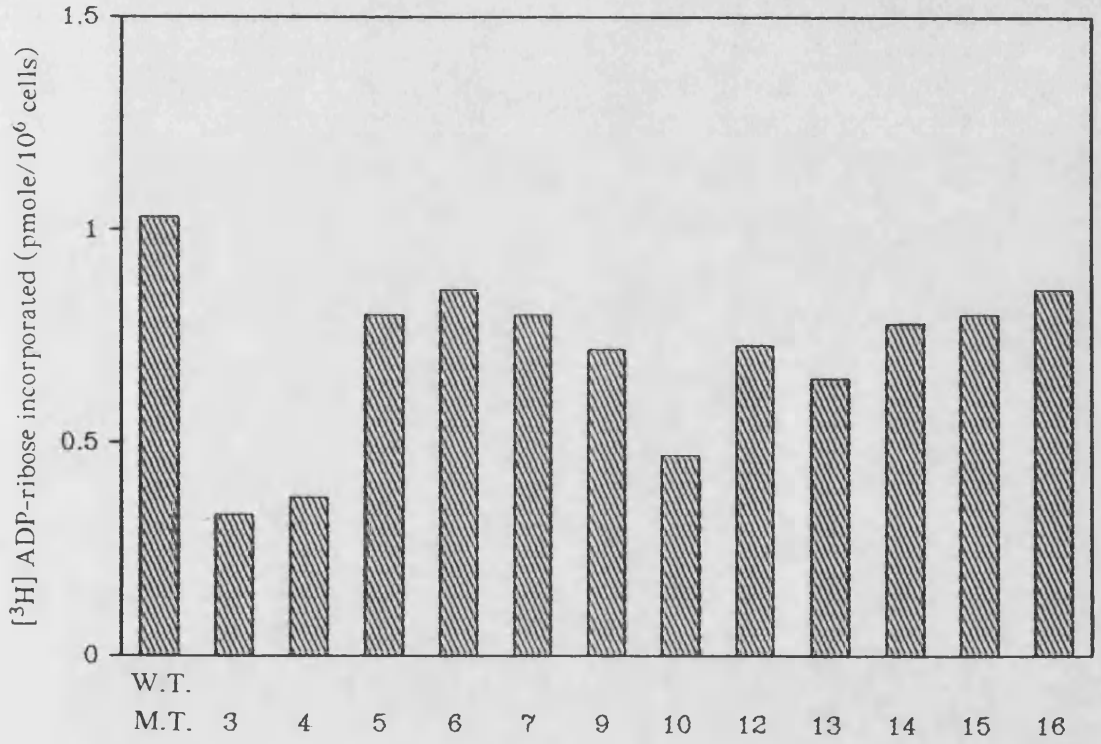


FIGURE 13.a Initial rate of poly (ADP-ribose) synthesis (pmole/min/10^6 cells) of wild-type and various mutant cell lines, measured as the incorporation of [^{3}H] ADPR from [^{3}H] NAD in permeabilized cells as presented in table11 (A).



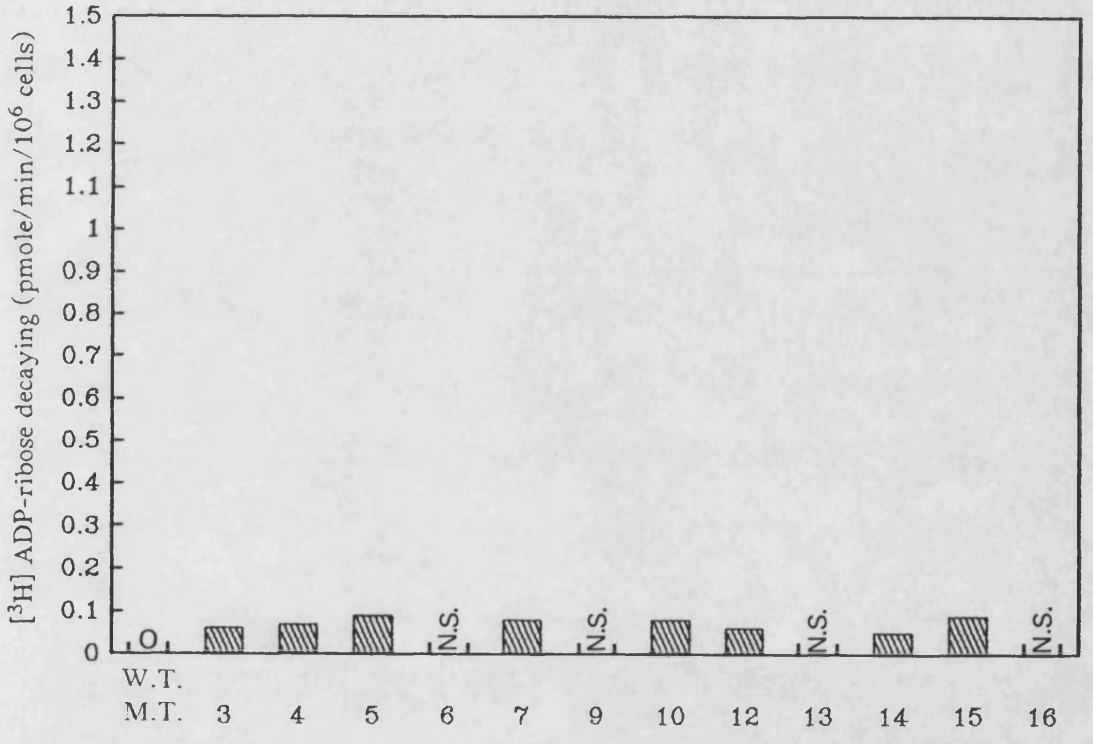
W.T.= Wild-type M.T.= Mutant

FIGURE 13.b The maximum amount of poly (ADP-ribose) systhesized by wild-type and various mutant cell lines during the plateau period (pmole/10^6 cells) measured as the incorporation of [^{3}H] ADPR from [^{3}H] NAD in permeabilized cells as presented in table 11 (B).



W.T.=Wild-type M.T.=Mutant

FIGURE 13.c The rate of decay of poly (ADP-ribose) after the plateau period in wild-type and various mutant cell lines (pmole/min/10^6 cells) measured as the degradation of poly (^{3}H-ADP-ribose) in permeabilized cells presented in table 11 (C).



W.T.= Wild-type M.T.= Mutant N.S. = Not significant

The rate of decaying was within the range of (0.05-0.09) pmole/min per 10^6cells (see figure 13 c).

DISCUSSION

The results show that all mutant cell lines possess ADPR transferase activity. The incorporation of radioactivity from [^{3}H] NAD into TCA insoluble products represents the net rate of ADP-ribosylation (ADPR transferase activity less poly (ADP- ribose) glycohydrolase and ADP-ribosyl protein lyase activities). The net rate of ADP-ribosylation of most mutant cell lines during the initial rates of reaction (except mutants 3 and 4) may be occuring by a similar process to that of wild-type cells. Moreover, most mutant cell lines (except 3, 4 and 10) produce a similar amount of polymer products during the plateau period to wild-type cells (figure 13, b). Both results indicate that the mechanism of ADP-ribosylation in wild-type and mutant cell lines are similar. It is not clear whether the ADPR transferase enzyme is identical in all cell lines or if the amount of enzyme is the same. As no significant differences were observed in the initial rates of reaction and in the maximum amount of polymers produced in all mutant cell lines (except mutants 3, 4, 10) it is assumed that the enzyme in most mutant cells remain unchanged.

The ADPR transferase of mutants 3 and 4 may possess more initiation sites than

ADPR transferase of wild-type cells. Multiple initiation sites were first reported by Ueda *et al.* (1980) and was supported by Kristen *et al.* (1985) and Bauer *et al.* (1986) who showed that mono ADP-ribose adducts were synthesized when isolated rat liver nuclei were incubated with low levels of NAD (nanomolar concentration). If ADPR transferase in mutants 3 and 4 contains multiple initiation sites, the major products shown on table 11 (over 80%) are probably mono ADP-ribose-enzyme protein initiators (Bauer *et al.*, 1986) which are very unstable (Yoshihara *et al.*, 1977; Ueda *et al.*, 1980) making measurement of the product difficult.

That ADPR transferase in mutants 3 and 4 may possess multiple initiation sites is concluded from the increasing initial rates of reaction of ADPR transferase when permeabilized cells of mutant 3 were incubated with increasing concentrations of NAD (table 12, figure 15). It is interesting that the lower activity of ADPR transferase in mutant 3 compared to wild-type cells is only observed when a low level of NAD concentration was used (ie 0.86 μM). This concentration was used in all mutant cell lines (figure 12 a-d). ADPR transferase activity of mutant 3 is apparently higher than wild-type cells when the NAD concentration used was 4.26 μM or greater (see table 12). This could be explained by the many initiator adducts (mono- ADP-ribose-enzyme protein) being further elongated to form polymers in higher concentrations of NAD, as it has an unlimited supply of substrate.

The lowering of the initial rates of poly (ADP-ribose) synthesis observed in mutants 3 and 4 may reflect the increase in apparent Km of the enzyme for NAD. The apparent Km of ADPR transferase for NAD of mutant 3 and wild-type cells together with their Vmax were estimated by the method of Lineweaver and Burk (1934) and it is shown in figure 14 and table 13. The kinetic parameters of the reaction indicates that the apparent Km for NAD of wild-type cells is 32 μM and

the Vmax is approximately 16 pmole/min/ 10^6 cells. In contrast, the apparent Km for NAD of mutant 3 is 3 times higher (106 μM) and the Vmax, also 3 fold higher, is equal to 52 pmol/min/10^6 cells. This kinetic behavior of the polymer further suggests that the enzyme in mutant 3 is preferentially catalyzing the initiation [mono-(ADP-ribosyl) ation] of protein rather than the elongation [poly-(ADP-ribosyl) ation] of proteins when a lower level of NAD was used (ie 0.85 μM). Hence low ADPR transferase activity is observed in mutant 3 as compared with wild-type cells (table 11).

The difference in NAD affinity of ADPR transferase in mutant 3 and wild-type cell lines may also be due to the different level of enzyme-associated DNA defined as sDNA which is DNA attached to ADPR transferase. It was found that the Km for NAD of ADPR transferase purified from calf thymus varies from 40 μM to 216 μM which corresponds to the amount of sDNA present which was 10.5 to 1.5 ng respectively (Neidergang*et al.*, 1979). The exact catalytic mechanism which makes DNA necessary for poly (ADP-ribose) synthesis is not known but there are indications that one of its roles may be in ADP- ribose chain elongation (Bauer and Kun, 1985).

Another reason for the differences in the ADPR transferase activity of mutants 3 and 4 as compared to wild-type cells may be due to multiple forms of enzyme as proposed by Dungan *et al.* (1974). These authors suggest that ADPR transferase exists in multiple forms based on the differences in properties of crude soluble and particulate enzyme preparations. It has also been suggested that there might be more than one ADPR transferase present inside the cells as two Km values were observed in HeLa cells. Km values of 42 μM NAD in S phase nuclei and 75 μM NAD in G_2 phase nuclei were found for poly (ADP-ribose) synthesis (Kidwell and

TABLE 12 Summary of poly (ADP-ribose) synthesis in wild-type and mutant 3 measured as the incorporation of [^{3}H] ADPR from [^{3}H] NAD in permeabilized cells at various concentrations of NAD, as described and presented in figure 16 a & b (results obtained from 3 independent experiments).

NAD	wild-type cells	Mutant 3 cells	
	(A)	(A)	(B)
(μM)	pmole/min/ 10^6 cells	pmole/min/ 10^6 cells	pmole/min/ 10^6 cells
4.26	1.40±0.08	2.0±0.32	0.13±.02
14.54	5.20±0.78	6.0±2.10	0.27±.09
35.06	8.24±1.70	12.5±1.50	0.34±.08
69.26	11.02±2.00	20.2±6.25	N.S.
137.66	13.75±2.10	28.0±1.12	N.S.

N.S. = Not significant

(A) The initial rate of poly (ADP-ribose) synthesis (pmole/min/10^6 cells)

(B) The rate of decay of poly(ADP-ribose) after the plateau period (pmole/min/10^6 cells)

TABLE 13 Apparent Km for NAD of ADPR transferase and Vmax of poly (ADP-ribose) synthesis in wild-type and mutant 3 cell lines determined by the method of Lineweaver and Burk as shown on figure 14 (results obtained from 3 determinations).

	Km	Vmax
	μM	pmole/min/10^6cells
Wild-type	31.23 ± 2.05	16.23 ± 3.4
Mutant	106.66 ± 2.88	52.20 ± 12.2

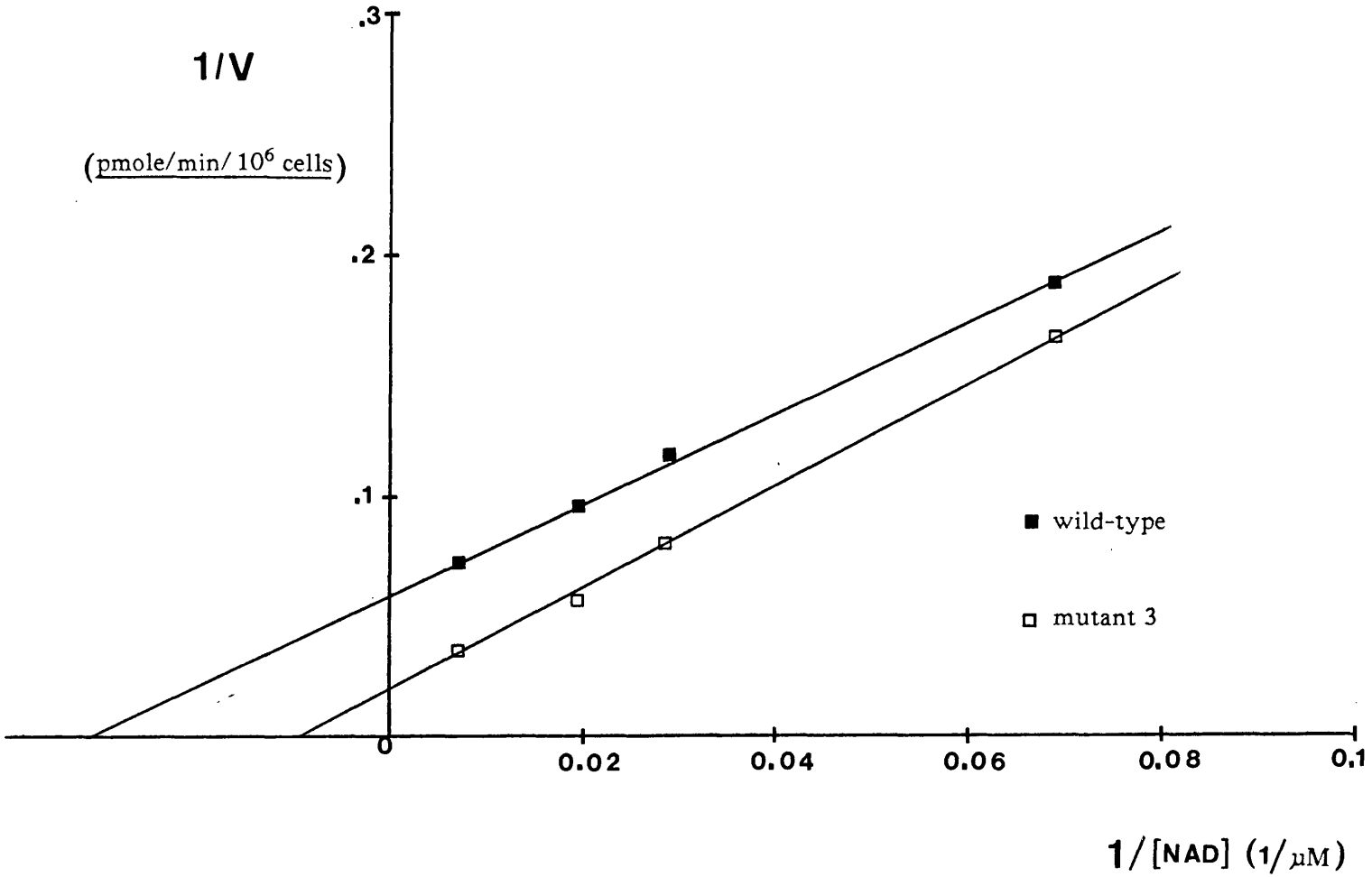
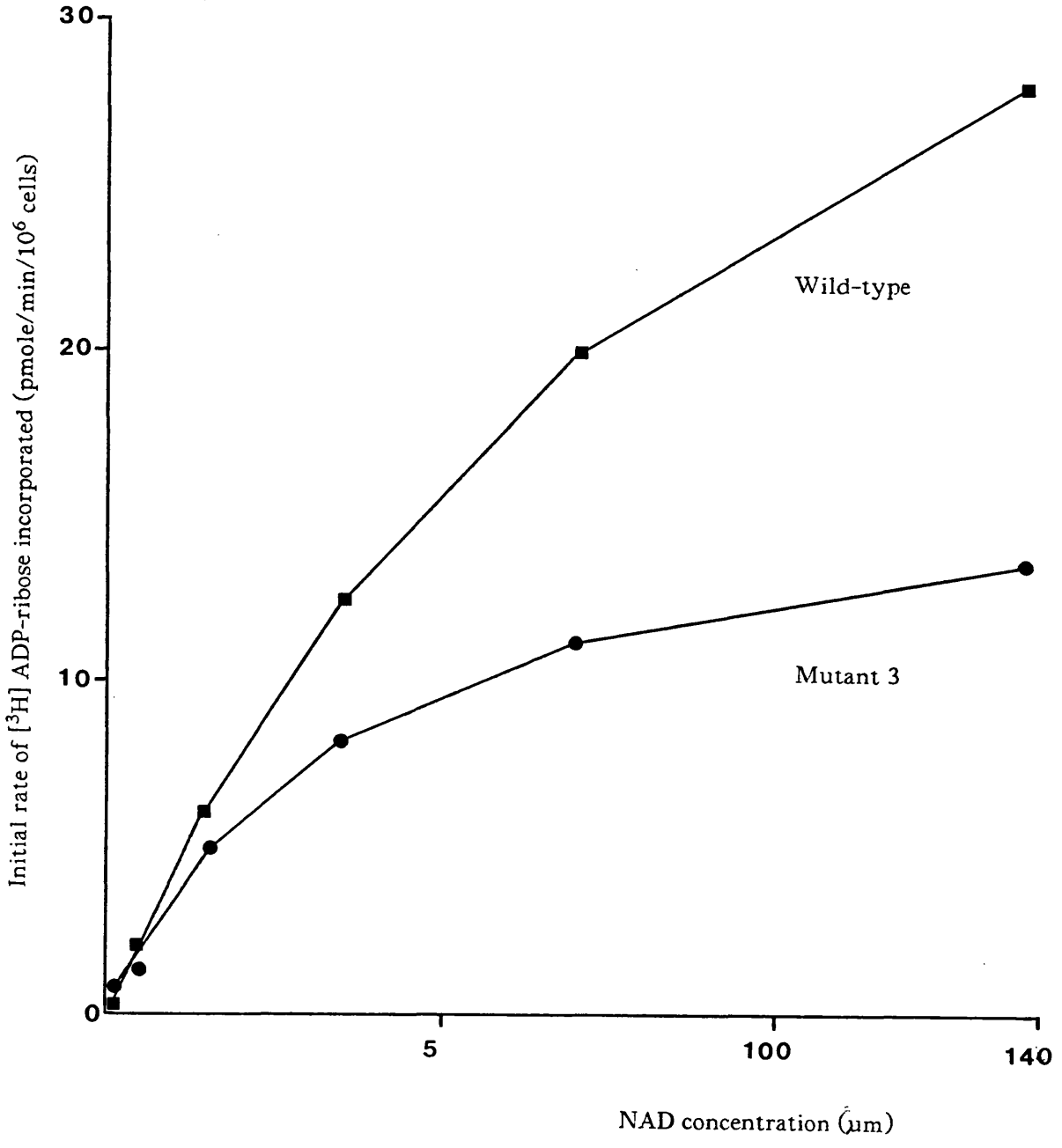


FIGURE 14 Km determination for NAD in wild-type and mutant3 cells.

FIGURE 15 Initial rates of poly (ADP-ribose) synthesis (pmole/min/10^6 cells) of wild-type and mutant 3 cells at various concentrations of NAD measured as the incorporation of [^{3}H] ADPR from [^{3}H] NAD in permeabilized cells. See also table 12.



Burdette, 1974). The above factors may contribute to the differences in the ADPR transferase activity observed in mutants 3 and 4.

The ratio of acceptor protein to DNA is also found to influence ADPR transferase such that the peak of ADPR transferase activity occurs at H1 : DNA ratio of between 1.0 and 2.0 and the enzyme was most active when the ratio was between 1.5 and 2.0. The ADPR transferase activity dropped rapidly at ratios outside this range (Carter and Berger, 1981a).

Evidence that DNA content in the mammalian cell lines influences ADPR transferase activity has been reported by Chatterjee and co-workers (1987). They found that one of their variant cell lines [11(81)1], which has the lowest enzyme activity compared to its wild-type and other variant cell lines, also has the highest DNA content per cell. The DNA content per 10^6 cells in variant 11(81)1, is also 2 fold higher than its wild-type. Moreover, ADPR transferase activity in variant 11(81)1 cells was 37% of the ADPR transferase measured in the wild-type cells. Such a result is similar to mutant 3 and wild-type cells (as shown on table 11) where ADPR transferase activity of mutant 3 is approximately 40% of the ADPR transferase activity in wild- type cells (see also figure 13 a).

The difference in the maximum amount of poly (ADP-ribose) synthesized observed in mutants 3, 4 and 10 as compared to wild-type cells (figure 13 b) may be due to the different levels of various acceptor proteins present in the cells. It was reported

TABLE 14 DNA content in mutant 3 and wild-type cells.

Cell lines	μg DNA per 10^6 cells
Wild-type	15.83 ± 1.3
Mutant 3	29.66 ± 1.5

The ratio of DNA content in wild-type cells to mutant 3 = 1.0 : 1.9

The DNA content of mutant 3 and wild-type cells was measured according to Burton assay which is described in methods (chapter 3.12). Data shown above were obtained from 3 independent experiments each performed in quadruplicate.

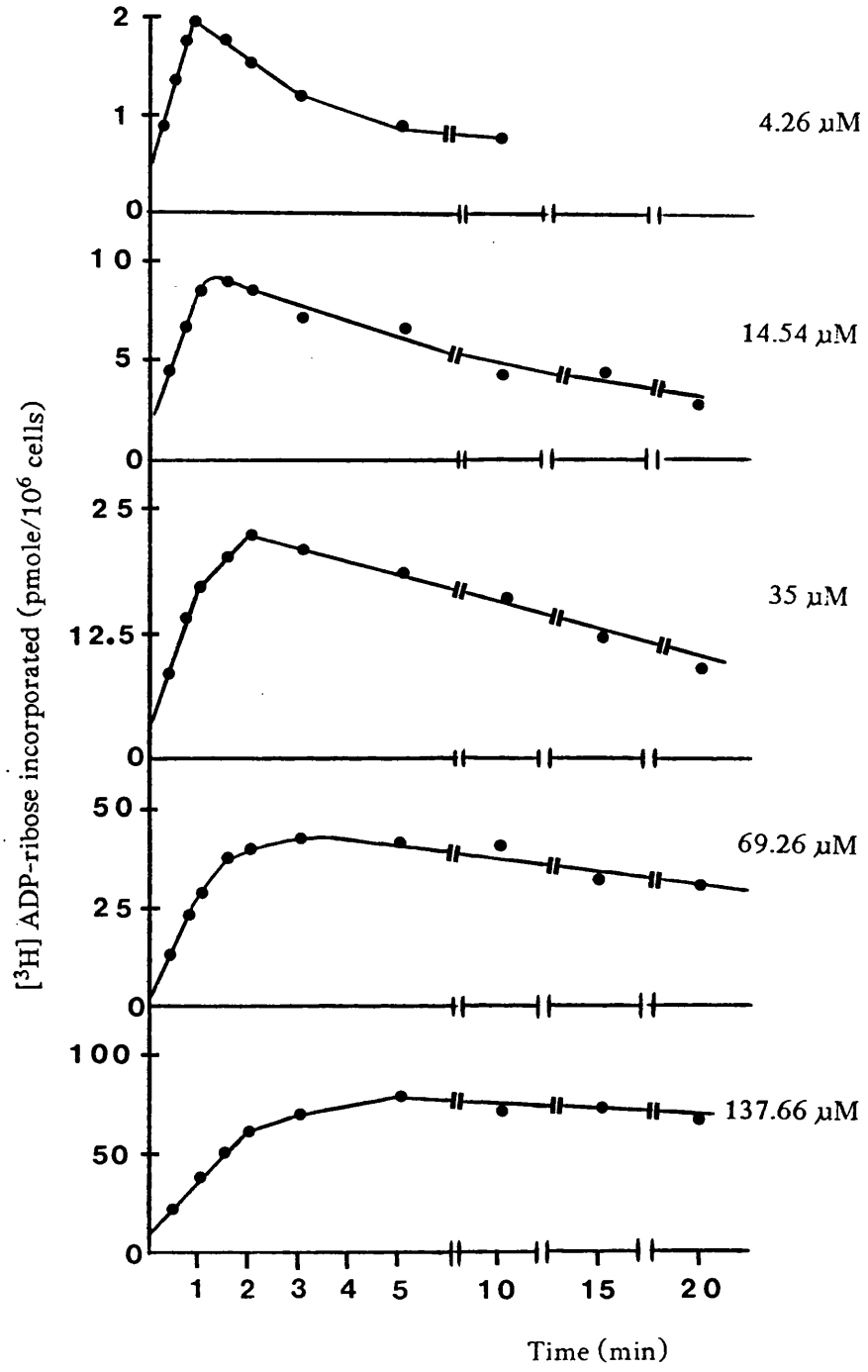
that the acceptor protein of ADP-ribosylation is dependent on NAD content. At higher concentrations of (>10 μM) NAD, histone H1 is the major acceptor protein of ADP-ribose in pancreatic nucleosomes (Aubin, 1982a, b) and at lower concentrations (<1 μM) the major acceptors are core histones and such accessibility of core histones to ADPR transferase was found to be dependant on the automodification state of the enzyme (Huletsky *et al.*, 1985).

The apparent decline or decay in poly (ADP-ribose) synthesis after the plateau in mutants, shown on figure 13 c, was probably complicated by an increase in the degradation of the polymer which could be produced by an increase in NAD glycohydrolase activity resulting in excessive substrate (NAD) consumption (Nakazawa *et al.*, 1968) or by poly (ADP-ribose) glycohydrolase or phosphodiesterase resulting in rapid decrease in poly (ADP-ribose). Poly (ADP-ribose) glycohydrolase cleaves the ribose-ribose bond between adjacent ADP-ribose units yielding an ADP-ribose monomer (Miwa and Sugimura, 1971). Phosphodiesterase hydrolyzes the phosphodiester bonds of poly (ADP-ribose) producing AMP, phosphoribosyl AMP and ribose 5'-phosphate (Shinshi *et al.*, 1976) hence an increase in activity in either of these two enzymes in mutant cells could cause rapid degradation of poly (ADP-ribose). The half-life of poly (ADP-ribose) was found to be quite short. A half-life of 1 min was reported by Hilz's group (Wielckens *et al.*, 1982). The sudden loss of poly (ADP- ribose) after 1-2 min in some of the mutant cell lines (mutants 3, 4, 5, 7, 10, 12, 14, 15 see figure 13 c) indicates that it is the result of an excess of degradation over synthesis caused by degrading enzymes, possibly by poly (ADP- ribose) glycohydrolase as phosphodiesterase is not considered to play a major role *in vivo* due to its lower specificity and affinity to the polymer (Miwa and Sugimura, 1982).

A detailed kinetic study of mutant 3 (as shown on table 12 and figure 16 a) shows that at higher doses of NAD, the product is still decreasing after 1-2 min of reactions. The rate of decay is 0.13, 0.27 and 0.34 pmole/min/10^6 cells for 4.26, 14.54 and 35.06 μM NAD respectively. The decrease in the rate of polymer synthesis has given further weight to the suggestion that poly (ADP-ribose) glycohydrolase in mutant 3 is in such excess that the time course does not slow the decay. However, at much higher doses of NAD (69 μM NAD) the rate of product decay in mutant 3 is very small and at 137 μM NAD, the product remains almost constant after 3 min reaction. This demonstrates that the constant net balance of synthesis and breakdown is probably due to the excess supply of NAD. In wild-type cells, at concentrations above 14 μM NAD (figure 16 b) the increase in polymer synthesis is observed for 5 min followed by a plateau, which remains for up to 20 min. This demonstrates that the net balance of synthesis and breakdown is achieved in wild-type cells at a much lower concentration of NAD (ie at 14 μM for wild-type and at 69 μM for mutant 3).

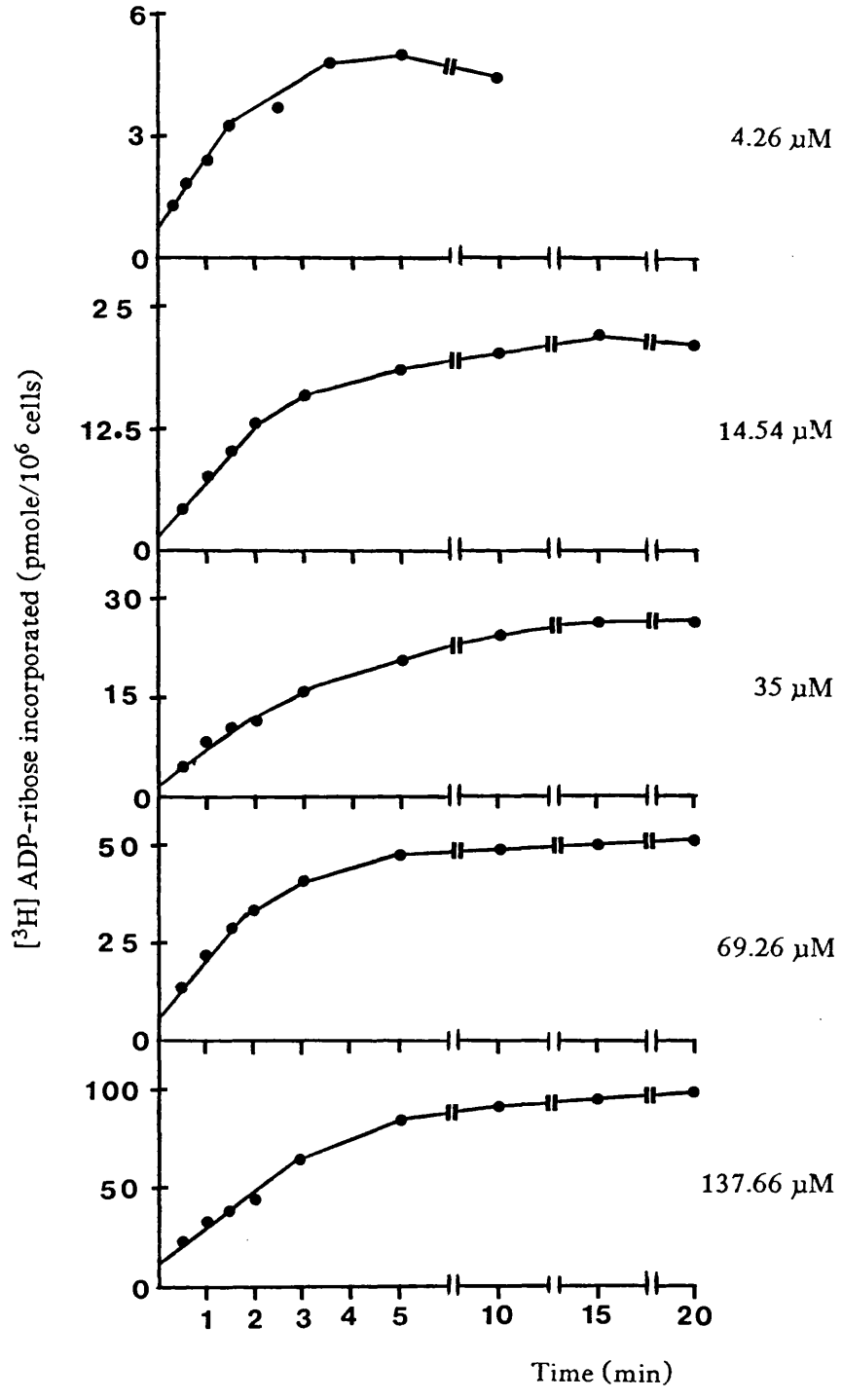
It can be concluded that all mutant cell lines possess ADPR transferase and that most mutant cell lines have a similar enzyme activity to wild-type cells. The lower ADPR transferase activity observed in mutants 3 and 4 are probably due to the various reasons previously described. Furthermore, the Km for NAD and the Vmax value in mutant 3 are shown to be higher than those obtained for wild-type cells. This kinetic parameter indicates that ADPR transferase in mutant 3 is different from ADPR transferase in wild-type cells. There is evidence that most mutant cell lines possess larger amounts of degrading enzymes as shown by the rate of decay of poly (ADP- ribose) after the plateau as shown in figure 13 c.

FIGURE 16.a Time courses of poly (ADP-ribose) synthesis of mutant 3 cells measured as the incorporation of [^{3}H] ADPR from [^{3}H] NAD in permeabilized cells.



Cells ($0.5x10^6$) were incubated with various NAD concentrations of 4.26, 14.54, 35.06, 69.26 and 137.66 uM with radioactivity specificity activity of 5.4, 1.6, 0.67, 0.33 and 0.17 mCi/µmole NAD respectively. Total volume of 250 µl was carried out at 26 deg.c. At various time intervals, an aliquot of 25 µl was spotted on Whatman filter paper disc (presoaked in 20% (w/v) TCA). Discs were washed and radioactivity determined according to methods described in chapter 3 (seetion 7 and 8).

FIGURE 16.b Times courses of poly (ADP-ribose) synthesis in wild-type cells measured as the incorporation of [^{3}H] ADPR from [^{3}H] NAD in permeabilized cells in various concentrations of NAD as described in figure 16.a.



5.2 NAD BIOSYNTHESIS IN MUTANT AND WILD-TYPE CELL LINES USING [^{14}C] NICOTINAMIDE AS PRECURSOR.

The conversion of nicotinamide to NAD has been reported to occur in various ways. It can enter the nicotinic acid pathway after deamination (Preiss and Handler, 1958) or it can be converted to NAD via nicotinamide mononucleotide as an intermediate (Dietrich *et al.*, 1966). It was shown that under the conditions normally used for culture, the net biosynthesis of NAD proceeds from nicotinamide by a single pathway with nicotinamide mononucleotide as the only intermediate (Jacobson *et al.*, 1979a). This pathway was demonstrated in L1210 cells in this laboratory. It was shown that nicotinamide is taken up by L1210 cells and incorporated into nicotinamide nucleotide of which 90% is converted into NAD (Al -Muhtaseb, 1985). This finding is in general agreement with those of Hillyard *et al.* (1973) using BHK 21/13 fibroblasts, murine 1921 and human D98/AH2 and Jacobson and Narasimham (1979) using mouse 3T3 cells. Some mammalian cells can also synthesize NAD from nicotinic acid and trytophan (Hillyard *et al.*, 1973). However, these NAD precursors are primarily utilized by the liver, which converts them to nicotinamide and releases the product into the systemic circulation (Bernofsky, 1980).

ADPR transferase is the main NAD degrading enzyme, exclusively located in the nucleus (chapter 1.1.3). In this process of poly (ADP-ribose) synthesis, nicotinamide is regenerated and converted back to NAD via the sequential action of NMN pyrophosphorylase and NAD pyrophosphorylase in a series of reactions that consumes 5 - phosphoribosyl-1-pyrophosphate (PRPP) and ATP (see figure 1).

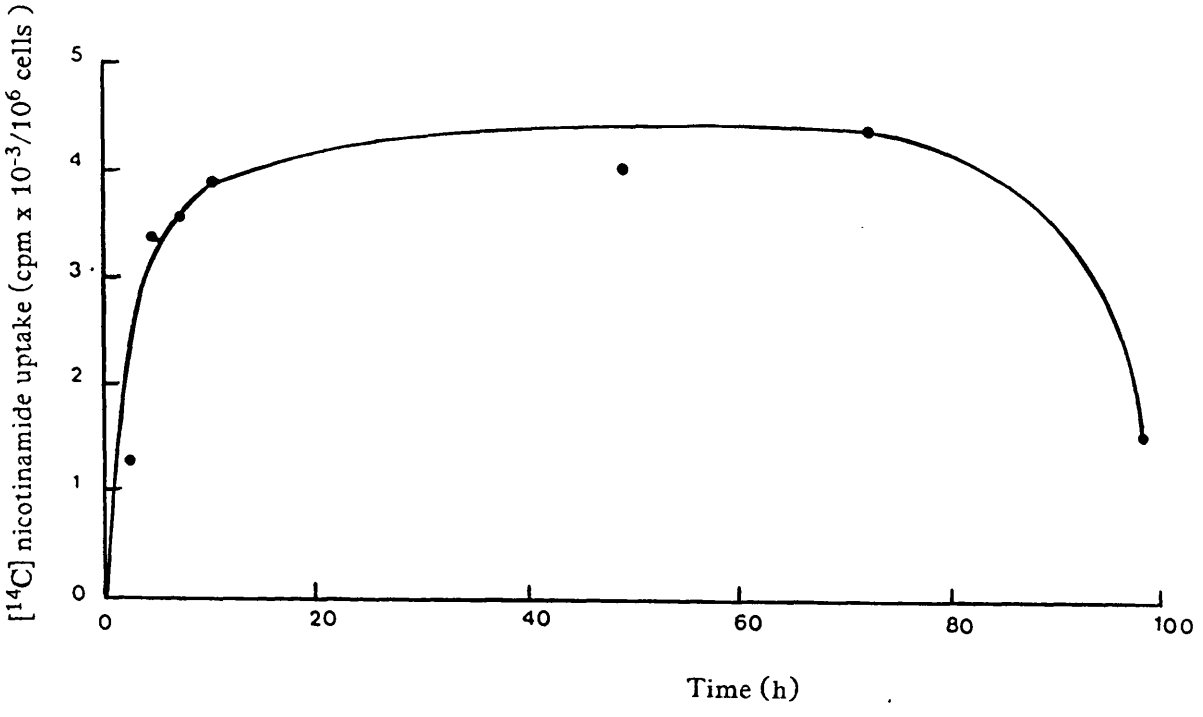
In this experiment, NAD biosynthesis in various mutant cell lines was determined using [^{14}C] nicotinamide as a direct precursor. This investigation was carried out to ascertain whether NAD is synthesized normally via nicotinamide, in various mutant cell lines. If mutation occurs at one of the enzymes responsible for this process results obtained should indicate this possibility when they were compared with wild- type cells.

It was shown earlier in this laboratory that wild-type L1210 cells labelled with low levels of [^{14}C] nicotinamide reached equilibrium labelling at 10 h of incubation and remains in equilibrium for up to 72 h and then decreases (see figure 17). Various mutant and wild-type cell lines were therefore labelled with [^{14}C] nicotinamide for a period of 48 h as this should ensure that all cell lines have reached their maximum uptake.

Various mutant and wild-type cell lines were prepared as described in methods (chapter 3.4.4). A culture bottle of cells was grown in normal RPMI, at cell density of 5-7x10^4 cells/ml, in the presence of [^{14}C] nicotinamide (0.5 μCi in 50 ml culture) and left undisturbed in the CO_2 incubator.

After 48 h, the extracellular [^{14}C] nicotinamide was removed. First the medium was carefully aspirated into a small volume (about 10 ml) leaving cells undisturbed and then the cells were collected by centrifugation (1000 rpm for 5 min) and the supernatant removed. The pellet was resuspended in cold 15 ml PBS and then centrifuged as before. This washing procedure was carried out a number of times and the radioactivity of the supernatants was counted. It was shown that the extracellular radiolabel is reduced to background levels after 4 washings (see table 15). Therefore, all cell lines labelled with [^{14}C] nicotinamide were always washed

FIGURE 17 [^{14}C] Nicotinamide incorporation into wild-type L1210 cells (Al - Mutaseb, 1985).



The uptake of labelled [^{14}C] nicotinamide in wild-type L1210 cells. These were grown in RPMI medium containing [^{14}C] nicotinamide. At the time shown, the cells were washed 4 times(4×15ml RPMI) and radioactivity counted as described in chapter 3.7.

4 times with 15 ml PBS. Cells were collected by microcentrifuge (high speed for 1 min) and ethanol was added to give a final solution of 50% (v/v). Finally, the suspension (about 200 µl) was sonicated at 15 w for 15 sec with an ultrasonicator using a probe of diameter 4.5 mm. This procedure was repeated 3 times after 1 min intervals. The mixture was recentrifuged (high speed/1 min) and the supernatant was assayed for NAD content. The method of thin layer chromatography (t.l.c.) in butanol system was used to separate radioactive NAD and nicotinamide (see chapter 3.8). This system is so sensitive that it will separate nicotinamide and nicotinic acid from pyridine nucleotides (NAD, NADH, NADP). R_f values for nicotinamide and nicotinic acid are 0.71 and 0.31 respectively, whereas pyridine nucleotides remain at the origin. Radioactivity from various spots on t.l.c. plates (see figure 18) were expressed as percentage fraction of total radioactivity of each plate. Results are summarized and presented in table 16.

RESULTS

The t.l.c. plates of various mutant cell lines were developed and the radioactivity determined. It was shown that there were only 2 spots which exhibited radioactivity (see figure 18). One was at the origin (spot A) which could be identified as a pyridine nucleotide (NAD, NADH and NADP) as it has R_f value of 0 and the other was nicotinamide (spot B) as it has R_f value of 0.71. The majority of radioactivity (> 85%) of total radioactive counts for various mutant and wild-type cell lines was found to be at the origin with the remaining of 10- 15% near to the top of t.l.c. plates. There was no trace of nicotinic acid on all plates developed.

Table 16 summarized the percentage fraction of [^{14}C] nicotinamide on t.l.c. plates, identified as NAD, NADH and NADP (R_f =0) and nicotinamide (R_f =0.71). Results

TABLE 15 Washing procedure to remove extracellular radiolabeled from mutant and wild-type cell lines.

Washing No	1st experiment	2nd experiment
1	700	810
2	80	70
3	40	30
4	15	10
5	0	0

Cells were labelled with [^{14}C] nicotinamide for 48 h and harvested by centrifugation (1000 rpm for 5 min). The supernatant was removed and the cells washed. A sample (200 μl) was taken from each supernatant and radioactivity was counted.

show that 90% of nicotinamide in wild-type cells was incorporated into pyridine nucleotides. Similar results were also observed for various mutant cell lines. In some mutant cell lines (mutants 9, 12, 14, 15 and 16) only 86% of nicotinamide was incorporated into pyridine nucleotides, but this small difference was considered to be insignificant.

DISCUSSION

The level of various nicotinamide metabolite(using [^{14}C] nicotinamide as precursor) in wild-type cells was determined by Al-Mutaseb (1985), using Dowex Ion-Exchange Chromatography. This method can separate nicotinamide plus NMN, NAD and NADH. Results obtained showed that the ratios of nicotinamide/NMN to NAD and to NADH is 3-5% : 86-90% : 3-5%. Furthermore, this ratio remains constant during the growth cycle of wild-type cells.

NAD biosynthesis of wild-type and various mutant cell lines are carried out in the same experimental procedure as Al- Mutaseb (1985). Moreover, results obtained for various mutant cell lines are similar to wild-type cells. This indicates that 90% of [^{14}C] nicotinamide is incorporated normally into nicotinamide nucleotides and that the majority of the product is likely to represent NAD as found in wild-type cells.

Although the pathways of NAD biosynthesis are not determined, the route of this process in mutant cells can be safely assumed to proceed from Nm→NMN→NAD as shown in the t.l.c. plates run in the butanol system (see figure 18); and as shown by other workers using 3T3 cells (Jacobson *et al.*, 1979 a) and wild- type L1210 cells (Al-Mutaseb, 1985). During the analysis of nicotinamide nucleotides, there is no

TABLE 16 Estimation of [^{14}C] nicotinamide incorporated into NAD and nicotinamide nucleotides in various mutant and wild-type cell lines using thin layer chromatography (butanol system), (results obtained from 3 determinations).

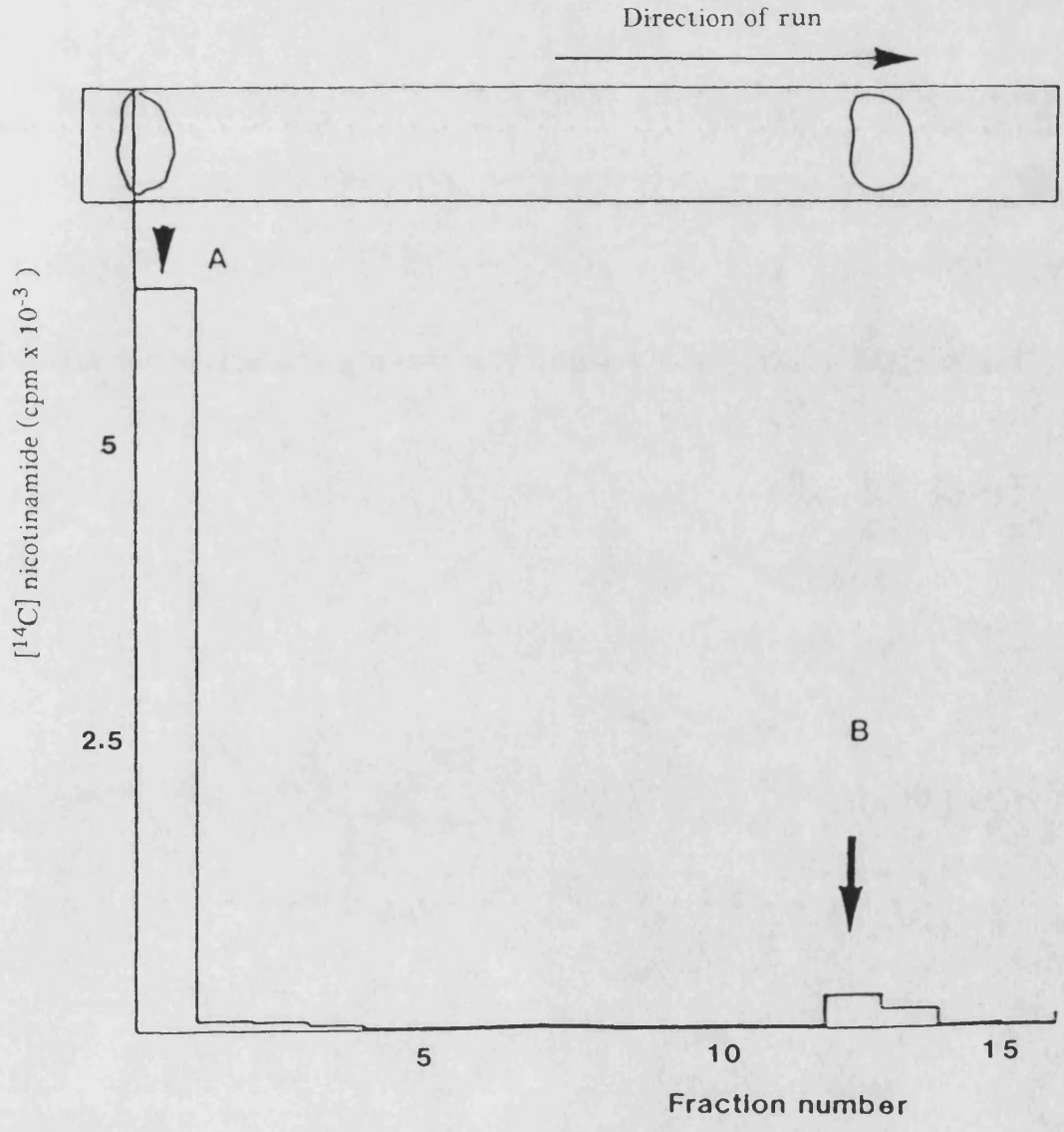
Cell lines	% radiolable on spot A NAD/NADH/NADP (R_f =0)	% radiolable on spot B Nicotinamide (R_f =0.71)
Wild-type	90.0 ± 0.8	10.0 ± 1.0
Mutant 3	89.0 ± 1.6	11.0 ± 1.7
4	90.0 ± 0.8	10.0 ± 1.0
5	89.0 ± 1.5	10.6 ± 2.0
6	91.6 ± 1.2	8.3 ± 2.0
7	91.0 ± 0.8	9.0 ± 1.0
9	86.5 ± 0.5	13.5 ± 0.5
10	91.1 ± 0.8	9.0 ± 0.6
12	87.7 ± 1.4	13.0 ± 1.0
13	90.3 ± 1.5	9.7 ± 2.5
14	86.6 ± 0.9	13.4 ± 1.3
15	86.0 ± 1.5	14.0 ± 1.2
16	88.0 ± 2.0	12.0 ± 1.5

Cells were labelled with [^{14}C] Nicotinamide for 48 h (0.5 μCi in 50 ml of culture medium), then washed 4 times to remove extracellular radiolabel. Finally, 50% (v/v) ethanol was added to cells, followed by sonication (3x15 sec). A sample of supernatant was analysed by t.l.c. using the butanol system and the radioactivity on t.l.c. plates was determined. Results expressed as percentage fraction of [^{14}C] nicotinamide obtained from each t.l.c. plates (see figure 18).

FIGURE 18 Radioactivity profile produced by chromatography of labelled mutant and wild-type cell lines in the butanol system (as described in table 16).

A = NAD/NADH/NADP

B = nicotinamide



evidence of the degradation of nicotinamide to nicotinic acid in all the cell lines tested. This also indicates that there is no active nicotinamide deaminase. The t.l.c. plates also show no evidence of nicotinamide conversion of N' - methylnicotinamide (Lee *et al.*, 1971). The t.l.c. butanol system would have detected nicotinic acid and any metabolites even as low as 0.01% of the total labelled pool. The possibility that any nicotinic acid formed might have been directed into NaMn→NaAD→ NAD in such a fast step that it could not be detected by t.l.c./butanol system was proved not to be the case in wild- type cells by Al-Mutaseb (1985). The analysis of radiolabelled nicotinic acid uptake had shown that it was not further metabolised to NaMn or NaAD.

The possibility that the [^{14}C] label of nicotinamide or nicotinamide nucleotides leaks out of the mutant cells during washing was considered to be very unlikely. All the nicotinamide in the form of nucleotides would not be able to pass through an intact cell membrane because of it being phosphorylated. If nicotinamide nucleotides were to leak out, only a tiny percentage of the total label inside the cell would probably diffuse out of the cells in its attempt to reach equilibrium with the extracellular enviroment. In cultured cell lines, nicotinamide generated during NAD catabolism is re-utilized preferentially for NAD synthesis and it was found that it did not equilibrate rapidly with extracellular nicotinamide pools (Hillyard *et al.*, 1981). It was also found that [^{14}C] label of nicotinamide and nicotinamide nucleotides leaks out of wild-type cells quite slowly and that after a period of 4 h, only 10% of the total [^{14}C] label inside the cells had diffused into isotope-free medium (Al-Mutaseb, 1985).

Cell membrane permeability of mutant and wild-type cell lines to 3-acetamidobenzamide is shown to function normally (chapter 4.2.3). Based on this

observation, it is assumed that the cell membrane permeability of mutant cell lines to nicotinamide and nicotinamide nucleotides would function in a similar manner to wild-type cells. Even if [^{14}C] label in mutant cell lines could leak out at a faster rate than wild-type cells due to a larger surface area, this is very unlikely to affect the results since the washing period is very short (30-40 min). The amount of [^{14}C] label that leaked out should, therefore, be very small.

It can be concluded that NAD is synthesized normally in mutant cell lines in the route of Nm→NMN→NAD as normally found in most culture cell lines.

5.3 THE ENHANCEMENT OF CYTOTOXICITY OF DMS BY 3-ACETAMIDOBENZAMIDE ON MUTANT AND WILD-TYPE CELL LINES

It has been demonstrated that treatment of cells with DNA damaging agents results in a rapid lowering of NAD levels with a concomitant increase in ADPR transferase activities (Whish *et al.*, 1975; Skidmore *et al.*, 1979; Durkacz *et al.*, 1980a). The increase in ADPR transferase activity may be brought about by either alkylating agents, irradiation or endonucleases. The common effect of all these agents is that they ultimately result in increased ADPR transferase levels in a dose dependent manner as observed by Durkacz *et al.* (1980a), suggesting that ADP-ribosylation may have a role in DNA repair. This hypothesis is supported by the finding that addition of inhibitors of ADPR transferase such as 3- aminobenzamide and 5-methylnicotinamide markedly increase the cytotoxicity of DNA damaging agents by preventing the rejoining of strand break and the resuming of replicative DNA synthesis (Durkacz *et al.*, 1980a; Durrant and Boyle, 1982). The above phenomena together with further work carried out by Shall's group have led to the inference that ADP-ribosylation regulates DNA repair, possibly in the ligation step (Creissen and Shall, 1982). It was reported earlier that nutritional depletion of the cellular content of NAD has been found to greatly reduce the rate of DNA rejoining in L1210 cells after treatment with DMS (Jacobson *et al.*, 1979b).

The enhancement of cell killing by ADPR transferase inhibitors on cells treated with DNA damaging agents is due to inhibition of nuclear ADPR transferase with a consequent inhibition of DNA repair. Mutant cell lines were therefore treated with DMS in the presence and absence of a non-toxic concentration of 0.5 mM 3-acetamidobenzamide (3-AAB). If one of the enzymes involved in DNA repair is

genetically altered, the cells surviving in mutant cell lines should have different capacities to survive compared to wild-type cells.

In this investigation, DMS was used as a DNA damaging agent. It is an electrophilic compound (Miller, 1970) which reacts with nucleophilic sites in DNA, particularly the N_7 of guanine and to a lesser extent, the N_7 of adenine (Lawley, 1966). The introduction of the methyl group at N_7 of guanine labilizes the glycosidic linkage between the N_9 of purine and C_1 of the deoxyribose, thus making the bond more susceptible to hydrolysis. Although the N_7 of guanine is not involved in hydrogen bonding, the methylation of DNA at this position leads to the production of apurinic sites which is followed by the production of single strand breaks in the polynucleotide backbone. Thus damage to DNA will result in helix distortion and the loss of function in biological processes such as replication, transcription and cell growth. Cells however possess the capacity to cope with limited damage to their DNA in the form of repair process, such that the damaged regions are enzymatically removed from DNA and replaced by a new sequence of intact nucleotide with the gap being closed by ligase action (Lehmann and Bridges,1977).

The survival assay in soft agar (see chapter 3.4.5) is used to determine the cytotoxicity of DMS and the enhancement effect (if any) of 3-AAB on various mutant cell lines. The cytotoxicity of DMS on wild-type cells resulted in cell death (50%) when treated with 50 μM DMS and a greater percentage of cell death (>95%) was observed when 3-AAB (at non-toxic concentration) was introduced to cells prior to DMS treatment (Sujareerat, 1985). The experiments were therefore carried out at 50 μM DMS as its cytotoxicity permits a greater observation of cell killing in the presence and absence of 3- AAB.

The main object of this investigation is to determine whether 3-AAB, at a non-toxic concentration, would enhance the cytotoxicity of DMS in various mutant cell lines. It was also considered that a single dose of DMS treatment (at 50 μM) and DMS with 3-AAB (0.5 mM) on each mutant cell line would be sufficient to give preliminary evidence on cell survival. If a cytotoxicity dose response curve of both treatments, with various concentrations of DMS, were to be investigated on all mutant cell lines, this would have taken a long time to achieve bearing in mind the number of cell lines tested and the length of time required for the colonies to form.

Mutant and wild-type cells were prepared (see chapter 3.4.4) and used during their log growth phase. Cell cultures were always divided into 2 bottles (5 ml each). One was treated with DMS only and other with DMS and 3-AAB. This procedure was taken to ensure that both treatment groups started at the same cell density so that the results obtained could be compared more accurately within the same cell line. For the combined treatment, 3-AAB was always added to cell cultures 10 min prior to DMS treatment and this was to ensure that 3-AAB was present during immediate repair after DNA damage. 3-AAB was also present in the soft agar during the colonies forming period, so its cytotoxic effect could be observed on the entire process of DNA repair. The process of DMS treatment and cell plating in soft agar was described in methods (chapter 3.4.5).

After 2-3 weeks, the colonies from each of the treatment groups of various mutant cell lines were counted and presented in table 17. The precentage increase in cell killing of DMS by 3-AAB is calculated using the equation below.

$$\frac{\text{(Colonies in DMS treatment - Colonies in DMS + 3-AAB) x 100}}{\text{Colonies in DMS treatment}}$$

RESULTS

Results in table 17 showed that DMS was toxic to all mutant cells. Very few cells (out of 100 cells plated) were capable of repairing damage to its DNA and resume a normal division to form colonies. Less than 10 colonies were observed for most mutant cell lines treated with DMS alone. Some mutant cell lines (eg mutants 9, 10, 13, 14, 15 and 16) show a greater number of cell survival in which 20-40 colonies were observed. A greater number of colonies (50-80 colonies) were observed in wild-type cells.

Cell survival in DMS and 3-AAB treatment in all mutant cell lines were clearly shown to be lower than DMS treatment alone. Very few cells (less than 10 cells for mutant cell lines and 40 for wild-type cells out of 1000 cells plated) were capable of repairing their DNA damage in the presence of 3-AAB (ADPR transferase inhibitor) and therefore able to form colonies.

DISCUSSION

It is clearly demonstrated that 3-AAB enhanced the cytotoxicity of DMS in all mutant cell lines. The enhancement factor, presented as the percentage increase in cell killing of DMS by 3-AAB, shows that more than 90% of cells surviving in DMS treatment are killed when 3-AAB is present throughout the entire DNA repair process. This finding is similar to that of wild-type cells which demonstrates that 3-AAB was equally effective at potentiating the killing of mutant cells when treated with DMS. Results obtained from this experiments lend no support to the possibility that ADP-ribosylation is impaired or altered in the process of DNA

TABLE 17 Cell survival of mutant and wild-type cell lines following treatment with 50 μM DMS in the presence and absence of 0.5 mM 3-acetamidobenzamide (3-AAB) as described in methods (chapter 3.4.5) The values given are the mean of colonies ± standard deviation of 5 determinations.

Cell lines	Expt No	A	B	C
Wild-type	1	57 0 ± 6.2	15.0 ± 3.8	97
L1210 cells	2	81 2 ± 4.9	39.0 ± 5.8	95
	3	50 0 ± 5.6	10.0 ± 3.6	98
Mutant 3	1	12 0 ± 0.8	5.3 ± 0.5	96
	2	14 8 ± 3.5	5.0 ± 2.2	97
	3	3 3 ± 1.5	0.8 ± 0.5	98
Mutant 4	1	6 3 ± 0.9	6.8 ± 0.9	90
	2	2 0 ± 0.8	1.8 ± 0.9	92
	3	2 5 ± 0.6	1.0 ± 0.8	96
Mutant 5	1	2 0 ± 0.8	2.8 ± 0.5	87
	2	2 3 ± 0.7	1.0 ± 0.8	93
	3	1 5 ± 0.6	2.8 ± 0.5	90
Mutant 6	1	2 0 ± 1.2	2.5 ± 1.7	96
	2	7 5 ± 1.0	5.3 ± 0.5	95
	3	9 8 ± 1.0	9.5 ± 1.2	92

TABLE 17 (continue)

Mutant 7	1	5 0 ± 0.8	2.0 ± 0.8	96
	2	7 0 ± 0.8	3.3 ± 0.7	95
	3	2 5 ± 1.3	2.0 ± 1.4	92
Mutant 9	1	8 0 ± 2.7	0.8 ± 0.5	99
	2	22 8 ± 7.4	5.0 ± 0.8	98
	3	32 3 ± 5.1	11.8 ± 1.0	96
Mutant 10	1	30 0 ± 1.6	7.8 ± 0.9	94
	2	11 3 ± 3.3	7.0 ± 2.5	90
	3	21 0 ± 2.6	5.3 ± 1.0	97
Mutant 12	1	15 0 ± 1.5	1.5 ± 1.0	99
	2	9 0 ± 2.6	3.8 ± 0.5	96
	3	8 8 ± 2.0	7.3 ± 1.0	92
Mutant 13	1	42 0 ± 4.5	18.0 ± 2.5	96
	2	22 3 ± 5.0	17.3 ± 5.0	93
	3	17 5 ± 2.0	5.8 ± 0.9	97
Mutant 14	1	37 0 ± 5.2	30.2 ± 1.7	92
	2	44 5 ± 8.8	46.0 ± 5.5	90
	3	22 0 ± 2.4	11.5 ± 3.9	95
Mutant 15	1	4 5 ± 2.1	1.3 ± 0.9	93
	2	18 0 ± 2.2	3.3 ± 1.5	98

TABLE 17 (continue)

Mutant 16	1	13.8 ± 4.0	4.5 ± 2.0	97
	2	34.0 ± 8.1	15.0 ± 4.6	96

(A) Colonies obtained from $10^{\times}$ 100 cells plated following 50 μM DMS treatment

(B) Colonies obtained from 1000 cells plated following 50 μM DMS + 0.5 mM 3-AAB

(C) The percentage increase in cell killing of DMS by 3-AAB. Calculated from equation below:

$$\frac{\text{(Colonies in DMS only - colonies in DMS+3-AAB) x 100}}{\text{Colonies obtained in DMS only}}$$

repair in mutant cell lines.

The results obtained also agree with other published work (Durkacz *et al.*, 1980 a; Nduka *et al.*, 1980; Jacobson *et al.*, 1984), which demonstrated that processes sensitive to inhibition by nicotinamide analogues are required for cellular recovery from DNA damage. The studies of the cytotoxicity effects of monofunctional alkylating agents were carried out in variuos cell types, such as L1210 cells and human fibroblasts (Cleaver *et al.*, 1983; Durrant and Bolye, 1982; James and Lehmann, 1982) and various ADPR transferase inhibitors, for example 3-aminobenzamide and 5- methylnicotinamide. The degree of enhancement of cytotoxicity by such inhibitors is highly variable, depending upon cell types and DNA damaging agents.

As described in the introduction (chapter 1.1.5), the cytotoxicity effects of ADPR transferase inhibitors on various cell lines, reported by various workers, are not only due to their action on ADPR transferase. Some reports suggest that the inhibitors interfere with other metabolic pathways for example purine metabolism (Cleaver *et al.*, 1983) and the *de novo* synthesis of DNA purines (Cleaver *et al.*, 1983; Milan and Cleaver, 1984). Moreover, the enhancement effect of 3-AAB may be due to the effect on folate metabolism as 3-AAB was found to inhibit the incorporation of [^{14}C] formate (in wild-type cells treated with MNNG) into ATP and GTP. It may also have inhibited glycine incorporation into ATP and GTP and reduced both the incorporation of thymidine into DNA and the specific activity of the d TTP pool as suggested by Hunting *et al.* (1985)

3-AAB may affect mutant cells in the same functions as described above. Reports from various sources have also suggested that ADPR transferase is activated by

DNA damaging agents and is consequently inhibited by ADPR transferase inhibitors. Furthermore, the toxic synergism demonstrated in the reports is due to an inhibition of ADPR transferase (see chapter 1.1.9)

The increased cell killing observed in these mutant cell lines could be due to the influence on the cellular machinery that controls repair as observed in wild-type cells. Such investigations of the mechanism have proved difficult due to the great complexity of chromatin and of the subdivision of the cell cycle, especially the S phase. For the surviving colonies, DNA strand breaks caused by DMS are ultimately repaired. The delayed repair and persistence of breaks associated with the requirement of ADP-ribosylation is likely to have resulted in the increased frequency of genomic rearrangement as suggested by Lunec *et al.* (1984). They reported that more genomic rearrangement had taken place in murine lymphoma cells when ADPR transferase activity was inhibited by 3-AAB.

The cell survival of mutant cells following DMS and 3-AAB treatment also depends on the state of the individual cells during growth cycle. As cells are used during their log growth phase, the majority of cells are therefore in the state of dividing cells. 3-AAB is probably acting at the S phase of the cell cycle, resulting in cell cycle inhibition. This suggestion is based on the results of Jacobson *et al.* (1984) who found that an inhibitor of ADPR transferase (3- methoxybenzamide) delayed inhibition of DNA synthesis in MNNG treated exponentially dividing cells but not in the non-dividing cells (Jacobson *et al.*,1985 b). Various studies also suggest that dividing cells following DNA damage require an increased rate of ADP-ribosylation in order to complete the S phase and progress through the G_2 and M phase. When ADPR transferase inhibitors were presented, they prevented cells from recovering, especially from G_2 which eventually leads to cell death (Das *et al.*, 1984; Boorstein

and Pardee, 1984).

The cloning in soft agar, used for observing the enhancement effect of DNA damaging agents, has a limited use as only the end point (forming colonies) is adopted in determining the effect of inhibitor on cell recovery from DNA damage. Moreover, the cells capable of only 1 to 3 doublings are not counted as survivors, whereas measuring the overall rates of cell division in suspension medium includes this increase. The study of cells in soft agar therefore precludes many biochemical analyses, particularly at early times following DNA damage. This technique however is extremely sensitive because only individual cells capable of repetitive division proliferate to produce a visible colony.

Results from this experiment have indicated that the **cell survival** following DMS treatment in mutant cell lines and wild-type cells are similar. 3-AAB is shown to enhance the cytotoxicity of DMS with the same degree of enhancement in mutant and wild-type cell lines.

5.4 THE CYTOTOXICITY OF 3-AMINOBENZAMIDE (3-AB), 3-NITROBENZAMIDE (3-NB), 3-AMINOBENZOIC ACID (3-ABA), 3- NITROBENZOIC ACID (3-NBA) AND 3-ACETAMINOBENZOIC ACID (3- AABA) ON MUTANT AND WILD-TYPE CELL LINES

Results obtained so far have shown that the genetic alterations in mutant cell lines were not due to:

1) The cell membrane being impermeable to 3-AAB (chapter 4.2.3)

2) A defect in the ADP-ribosylation process. (ADPR transferase (see 5.1) and NAD synthesis (see 5.2) is shown to be present). Furthermore, the cell survival of mutant and wild-type cell lines following DMS treatment in the presence and absence of 3-AAB are shown to be similar. It was subsequently decided to see if these mutant cell lines (which are resistant to a toxic concentration of 3-AAB) were also resistant to the toxic concentrations of other ADPR transferase inhibitors. As results from various experiments have indicated that the mutation is not related to ADP- ribosylation, this implies that the mutation may be specific for the function related to the cytotoxicity of 3-AAB. This can be determined if mutant cell lines are not shown to be resistant to 3-AB and 3-NB.

3-Aminobenzamide (3-AB) and 3-nitrobenzamide (3-NB) are ADPR transferase inhibitors with Ki values of 2.6 and 9.6 μM respectively (Purnell and Whish, 1980). The acid analogues 3- aminobenzoic acid (3-ABA) 3-nitrobenzoic acid (3-NBA) and 3- acetaminobenzoic acid (3-AABA) which are not ADPR transferase inhibitors (Purnell and Whish, 1980) were also tested on mutant cell lines. The

major structural differences between 3-AAB, 3-AB and 3-NB are the acetyl, amino and nitro groups on the 3 substituted benzamide (see figure 4.b). The lack of the amide nitrogen and the ionization of the acid groups in the acid analogues together with their both being planar and very polar are the major differences between the parental amide and their acid analogues.

The cytotoxicity of 3-AB, 3-NB, 3-ABA, 3-NBA and 3-AABA was tested on wild-type cells. It was shown earlier that high concentrations of 3-AB and its acid analogue 3-ABA and 3-AABA are required in order to have any cytotoxic effect on wild- type cells. A much lower concentrations of 3-NB and its acid analogue (3-NBA) are required for a similar effect (Sujareerat, 1985). Wild-type cells (at $5x10^4$cells/ml) were treated with various concentrations of 3-AB, 3-ABA, 3-NB, 3-NBA and 3-AABA in the suspension medium (as shown in table 18). At various time intervals, normally every 24 h up to 96 h, cell density was counted. The doubling times of cells in various treatments were determined and presented in table 18.

Results showed that the concentrations of 14, 24, 3, 4 and 15 mM for 3-AB, 3-ABA, 3-NB, 3-NBA and 3-AABA, respectively, had a cytotoxicity effect on wild-type cells. Their cytotoxicity effects increased the normal doubling time of cells from 12 h to about 40 h. Moreover, the maximum cell density of $100x10^4$ cells/ml observed at the stationary phase (in the absence of inhibitors) was reduced to about $20x10^4$cells/ml. It was therefore decided to test the cytotoxicity of the above concentrations of various compounds on mutant cell lines. If mutant cell lines are to be resistant to 3-AB and 3-NB, the concentrations of both compounds used (which are toxic to wild-type cells) should not have any effect on mutant cell lines. The cytotoxicity of the acid analogues on mutant cell lines should also provide more

TABLE 18 Comparison of growth rates and the maximum cell density of wild-type cells in the presence of various concentrations of 3-aminobenzamide (3-AB), 3-aminobenzoic acid (3-ABA), 3-nitrobenzamide (3-NB), 3-nitrobenzoic acid (3-NBA) and 3-acetamidobenzaoic acid (3-AABA).

Compounds	[Con] (mM)	Doubling times (h)	Maximum cell density at the stationary phase (cells/ml)
RPMI	-	12.8 ± 1.5	100 ± 12.0
3-AB	10	25.0 ± 5.2	70 ± 8.0
	12	30.0 ± 4.2	55 ± 7.5
	14	42.6 ± 8.8	22 ± 5.3
3-ABA	20	20.0 ± 2.5	80 ± 7.8
	22	30.2 ± 5.0	50 ± 3.0
	24	41.5 ± 3.2	21 ± 4.5
3-NB	2	27.0 ± 3.0	52 ± 7.5
	3	43.3 ± 7.2	15 ± 3.6
3-NBA	2	18.0 ± 2.0	80 ± 10.0

TABLE 18 (continue)

	3	23.4 ± 1.2	60 ± 3.2
	4	40.0 ± 8.0	28 ± 3.5
3-AABA	10	18.0 ± 2.0	90 ± 10.0
	15	52.6 ± 8.3	14 ± 3.9

Wild-type cells at $5x10^4$ cells/ml were treated with various concentrations of the above compounds and at various time intervals (of every 24 h up to 96 h), cell density was determined. Cell cycle doublng times were determined from the slopes of the growth curves (6 determinations). Slopes were calculated from least square regression analysis of data.

evidence that the cytotoxic effects observed are not on ADPR transferase.

Various mutant cell lines were prepared (chapter 3.4.4) and subcultured during log growth phase into the media contaning 14, 24, 3, 4 and 15 mM of 3-AB, 3-ABA, 3-NB, 3-NBA and 3-AABA respectively. Cells were always started at $5x10^4$cells/ml in a culture bottle of 5 ml. Cell density was monitored at various time intervals, normally at every 24 h up to 96 h or longer. The maximum cell density at the stationary phase was also observed. The doubling times of mutant cell lines in various treatments were determined and presented in table 19. The ratios of doubling times of each cell line in the presence of inhibitors and acid analogues to its doubling time (within the same cell line) in the absence of various compounds are shown in table 20. These ratios are also presented as graphs in figure 19 (a-d). The summary of maximum cell density of all cell lines are presented in table 21 and as graphs in figure 20 (a, b, c).

RESULTS

Since the concentrations of various compounds were toxic to wild-type cells, the results obtained for mutant cell lines should provide sufficient evidence for the preliminary investigation. Results showed that the compounds tested were toxic to all mutant cell lines. The effects of their cytotoxicity on mutant cell lines vary from completely inhibiting cells from the process of cell division (no proliferation) to lengthening their doubling times. The cytotoxicity of 24 mM 3-aminobenzoic acid completely inhibited cell proliferation in mutants 6, 7, 9, 10 and 16. This observation was also observed in mutants 6, 9, 10, 12, 13, 14 and 16 for 3 mM 3-nitrobenzamide treatment, in mutants 6, 13 and 16 for 4 mM 3-nitrobenzoic acid treatment and in mutants 6, 7, 10, 12, 15 and 16 for 15 mM 3-acetamidobenzoic

acid treatment. For mutant cells that achieved the process of cell proliferation, however, at least 40 h or longer was required for cell population to double (see table 19)

Table 20 shows the increased time required for each cell line receiving various treatments to achieve their doubling cell density (cells/ml) (ie compared to its doubling time in the absence of treatment). ADPR transferase inhibitors and their acid analogues were toxic to wild-type cells such that cells require at least 3 times longer than their normal doubling time (no treatment) in order to achieve the doubling cell density. Most mutant cell lines also required at least 2 or 3 times longer in the presence of various compounds in order to achieve the doubling cell density. Mutant 3 was shown to be the least sensitive to the cytotoxicity of inhibitors and acid analogues. The factor (time) by which cells increased their doubling cell density in various treatments was only 1.5-2.0 times longer than its normal doubling time. An increase factor of time, less than 2, was also observed in mutant 4 for 3-nitrobenzoic acid, mutant 10 for 3-aminobenzamide treatment and in mutant 12 for 3- aminobenzamide and 3-nitrobenzoic acid treatment (see also figure 19 a, b, c and d).

Table 21 shows the maximum cell density of mutant cell lines in various treatments at the stationary phase. Results showed that the maximum cell density in most mutant cell lines was less than 50% of the maximum cell density obtained in normal media RPMI (see also figure 20 a, b, c). The maximum cell density of mutant 3 following 3-aminobenzamide and 3 acid analogues treatment was shownto be 50% or over of the maximum cell density in normal media RPMI. This observation is also observed in mutant 4 following 3-acetamidobenzoic acid (3-AABA) and 3- nitrobenzoic acid (3-NBA) treatment and in mutant 5 for 3-AABA

TABLE 19 Summary of the doubling times of mutant and wild-type cell lines in the presence of 14 mM 3-aminobenzamide (3-AB), 24 mM 3-aminobenzoic acid (3-ABA), 3mM 3- nitrobenzamide (3-NB), 4 mM 3-nitrobenzoic acid (3-NBA) and in 15 mM 3-acetamidobenzoic acid (3-AABA). (See figure 19 a, b, c, d).

Cell lines	Non-Treatment	3-AB	3-ABA	3-NB	3-NBA	3-AABA
Wild-type	12±2	42± 8	41± 3	43± 7	40± 8	53± 8
Mutant 3	29±1	42± 8	51± 9	58± 9	43± 3	51± 9
4	23±1	51± 4	54± 5	90±12	44± 7	50± 8
5	24±2	64±11	45± 9	70± 5	68±10	60±12
6	29±3	84+12	N.P.	N.P.	N.P.	N.P.
7	20±1	51±11	N.P.	85± 7	79±12	N.P.
9	23±1	59± 7	N.P.	N.P.	70±10	91±13
10	24±2	43± 6	N.P.	N.P.	79±12	N.P.
12	26±2	50± 4	53± 4	N.P.	41± 6	N.P.
13	20±1	60± 9	57± 8	N.P.	N.P.	69±12
14	24±2	70±10	80±11	N.P.	69±12	74± 9
15	20±2	44± 5	44± 3	69± 4	76±12	N.P.
16	22±2	44± 7	N.P.	N.P.	N.P.	N.P.

N.P. = Non-Proliferative

Vaxious cell lines at an initial cell density of $5x10^4$cells/ml were grown in the presence of the above compounds and cell density was counted every 24 h (to a maximum of 96 h). Cell cycle doubling times of various treatments were determined from the slopes of the growth curves (6 determinations). Slopes were calculated from least square regression analysis of data.

TABLE 20 Ratios of doubling time of each cell line (wild-type and mutant) in the presence of various compounds (dt+) to its doubling time in the absence of compounds (dt-) (as described in table 19;) presented as:-
(dt+)/(dt-). See also figure 19 (a, b, c, d).

Cell lines	dt(+AB) / dt(-)	dt(+ABA) / dt(-)	dt(+NB) / dt(-)	dt(+NBA) / dt(-)	dt(+AABA) / dt(-)
Wild-type	3.5	3.4	3.6	3.3	4.4
Mutant 3	1.5	1.8	2.0	1.5	1.7
4	2.3	2.4	3.9	1.9	2.2
5	2.7	1.9	2.9	2.9	2.5
6	2.9	0	0	0	0
7	2.6	0	4.3	4.0	0
9	2.6	0	0	3.0	4.0
10	1.8	0	0	3.3	0
12	1.9	2.0	0	1.6	0
13	3.0	2.9	0	0	3.5
14	2.9	3.3	0	2.9	3.0
15	2.2	2.2	3.5	3.8	0
16	2.0	0	0	0	0

dt = doubling time

TABLE 21 Maximum cell density at the stationary phase of mutant and wild-type cell lines in various treatments with 14 mM 3-aminobenzamide (3-AB), 24 mM 3-aminobenzoic acid (3-ABA), 3 mM 3-nitrobenzamide (3-NB), 4 mM 3-nitrobenzoic acid (3-NBA) and 15 mM 3-acetamidobenzoic acid (3-AABA).

Cell lines	Non-treatment	3-AB	3-ABA	3-NB	3-NBA	3-AABA
Wild-type	100±12	22±5	22±4	15±3	28±4	14±2
Mutant 3	41± 7	27±4	21±4	11±4	25±3	21±3
4	38± 6	16±3	13±2	10±2	26±5	20±3
5	37± 3	16±2	15±3	10±3	14±2	19±6
6	32± 6	10±3	N.P.	N.P.	N.P.	N.P.
7	46± 5	18±5	N.P.	10±2	10±2	N.P.
9	37± 3	15±4	N.P.	N.P.	14±2	11±3
10	40± 5	14±3	N.P.	N.P.	12±3	N.P.
12	41± 5	12±2	10±3	N.P.	18±4	N.P.
13	38± 5	13±3	12±3	N.P.	N.P.	10±2
14	34± 3	15±4	10±2	N.P.	10±2	15±2
15	33± 4	14±2	10±3	11±3	11±2	N.P.
16	35± 3	16±3	N.P.	N.P.	N.P.	N.P.

N.P. = Non-proliferative

Cells at an initial cell density $5x10^4$ cells/ml, were grown in suspension media RPMI containing various compounds as above and cell proliferation was monitored from 0 h up to about 96 h so that the maximum cell density could be counted (6 determinations). See also figure 20 (a-c).

FIGURE 19.a Doubling times of mutants 3,4,5 and wild-type cell lines in the presence of 14 mM 3-aminobenzamide (3-AB), 24 mM 3-aminobenzoic acid (3-ABA), 3 mM 3-nitrobenzamide (3-NB), 4 mM 3- nitrobenzoic acid (3-NBA) and in 15 mM 3- acetamidobenzoic acid (3-AABA) as deseribed in table 19. For the control, cells were grown in normal RPMI.

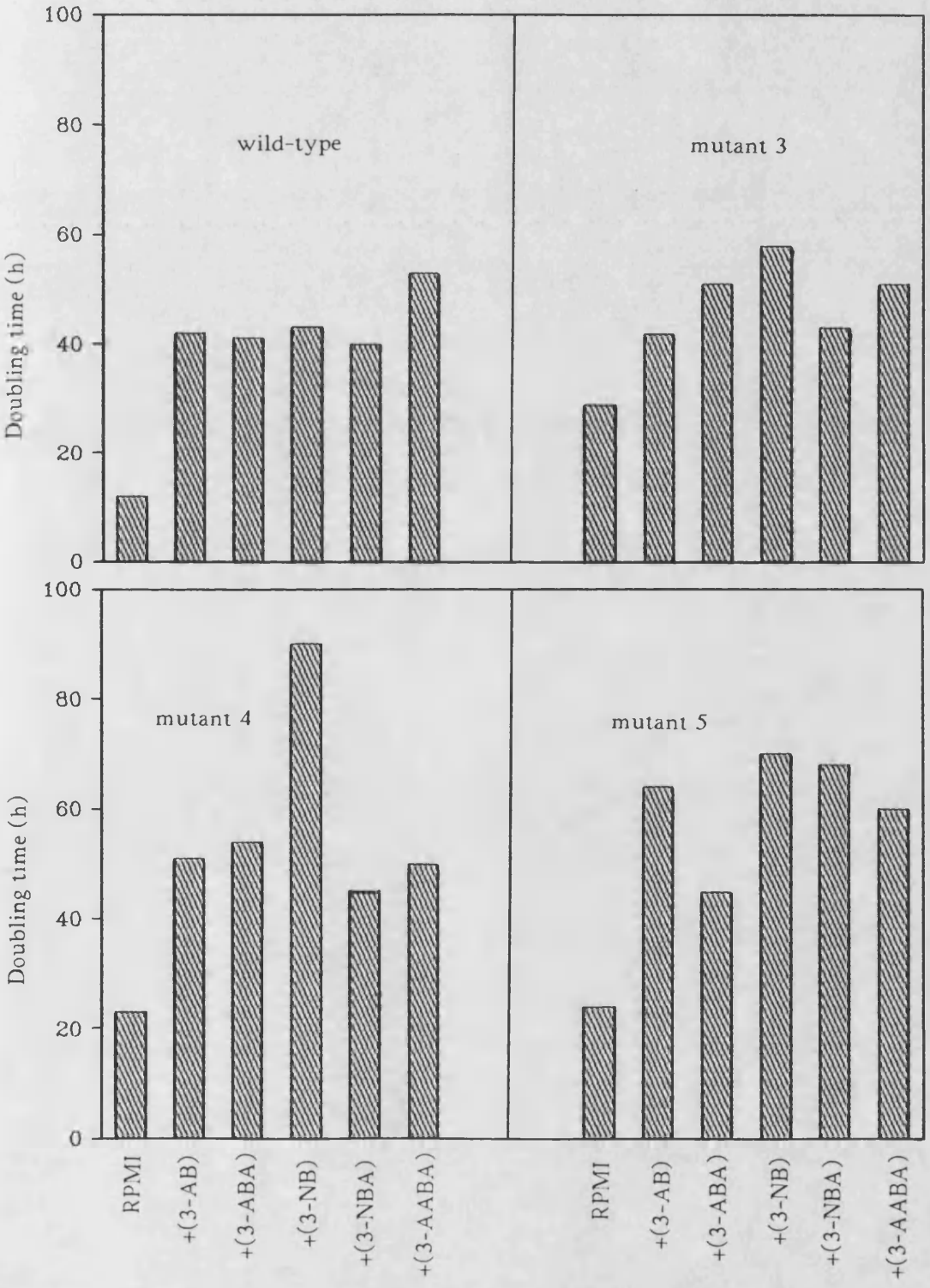


FIGURE 19.b Doubling times of mutants 6,7,9 and 10 in the presence of various compounds as described in figure 19.a.

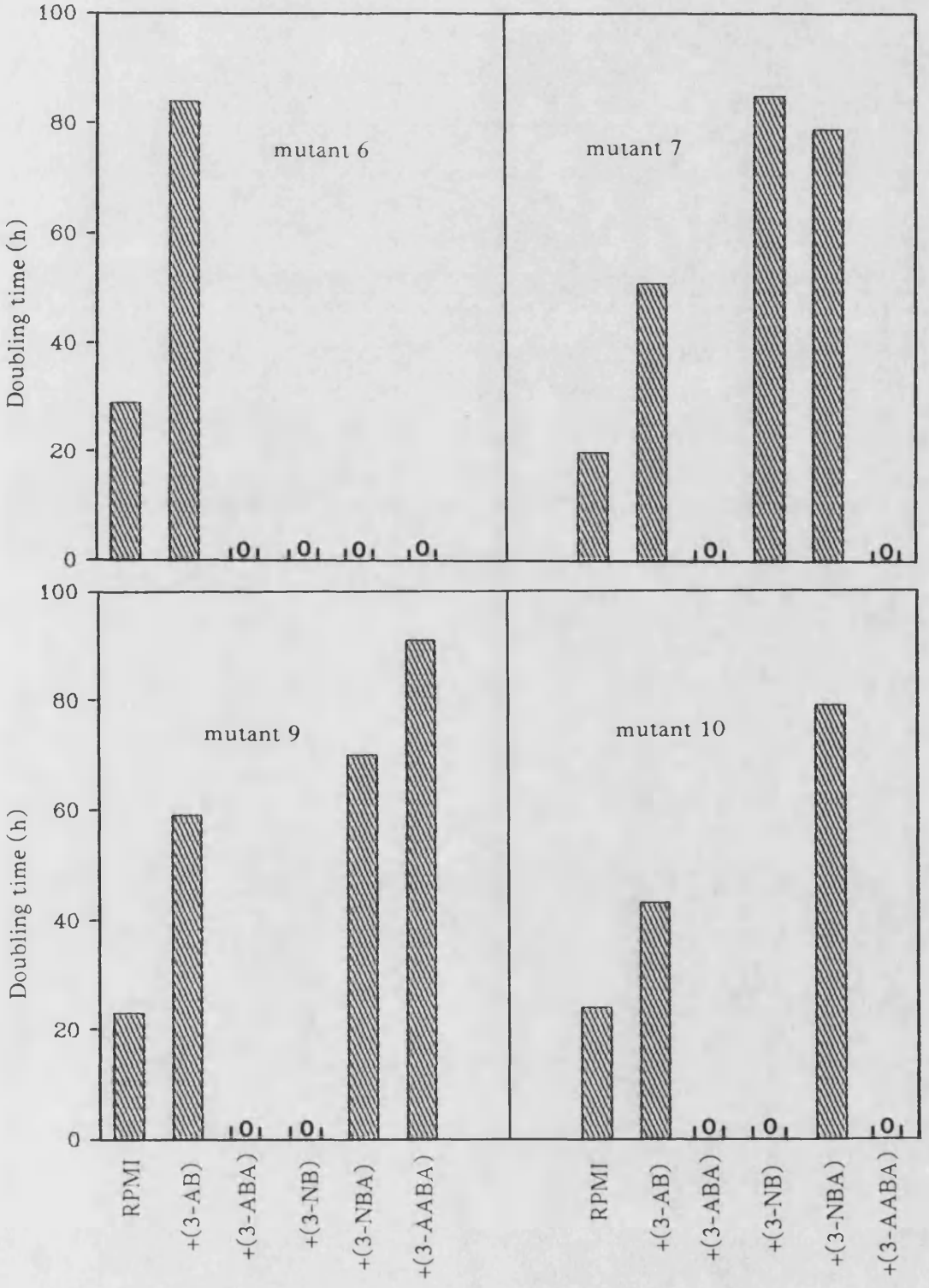


FIGURE 19.c Doubling times of mutants 12,13,14 and 15 in the presence of various compunds as described in figure 19.a.

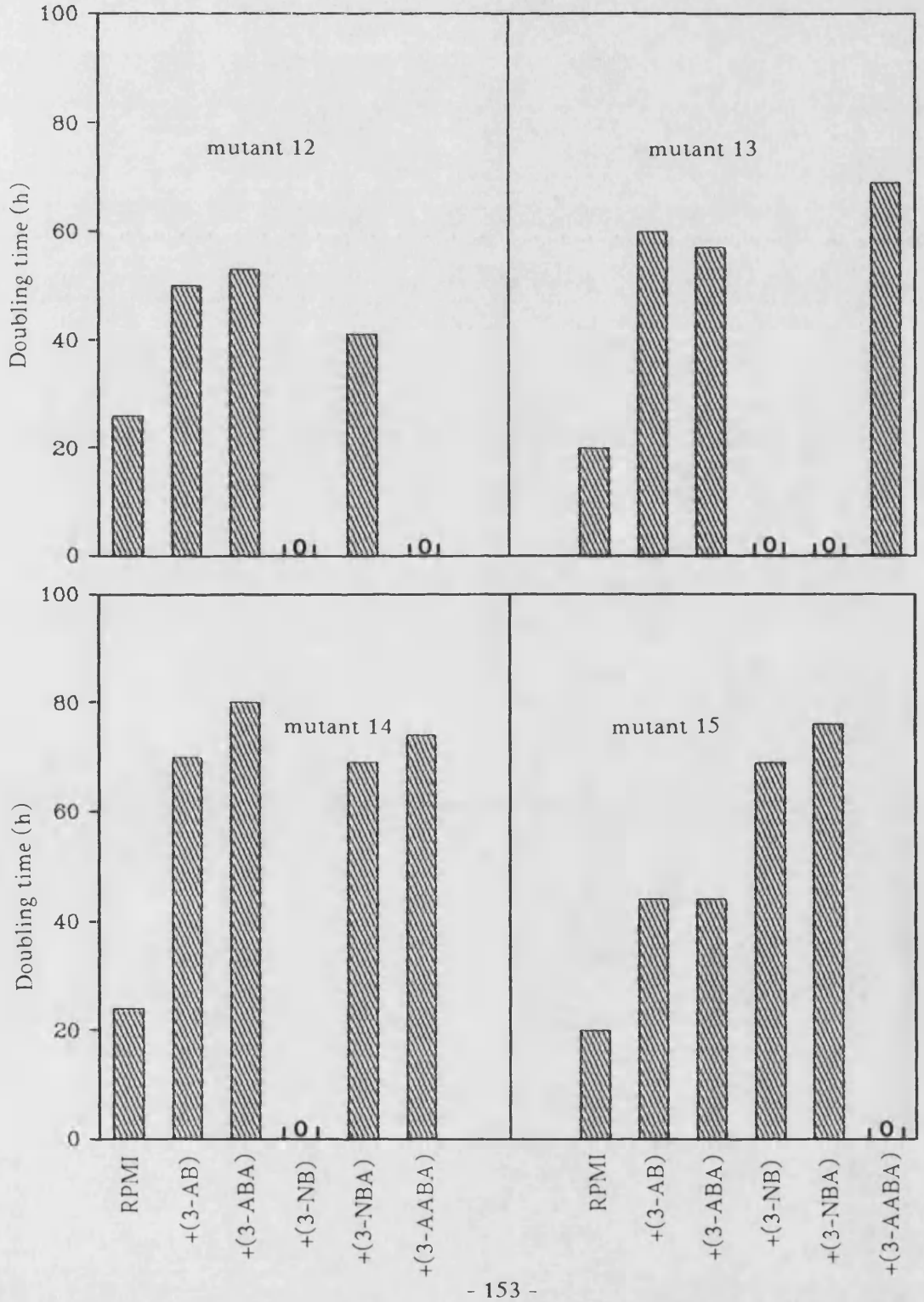


FIGURE 19.d Doubling times of mutant 16 in the presence of various compound as described in figure 19.a.

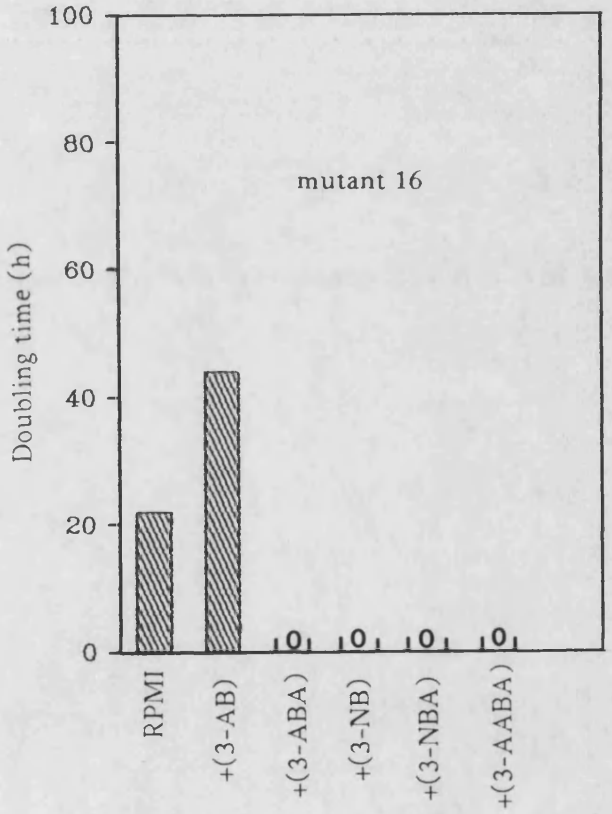


FIGURE 20.a Maximum cell density (cells/ml) at the stationary phase of wild-type and mutants 3,4,5 cell lines in the presence of 14 mM 3-AB, 24 mM 3-ABA, 3 mM 3-NB, 4 mM 3-NBA and 15 mM 3-AABA as deseribed in table 21. For the control, cells were grown in normal RPMI.

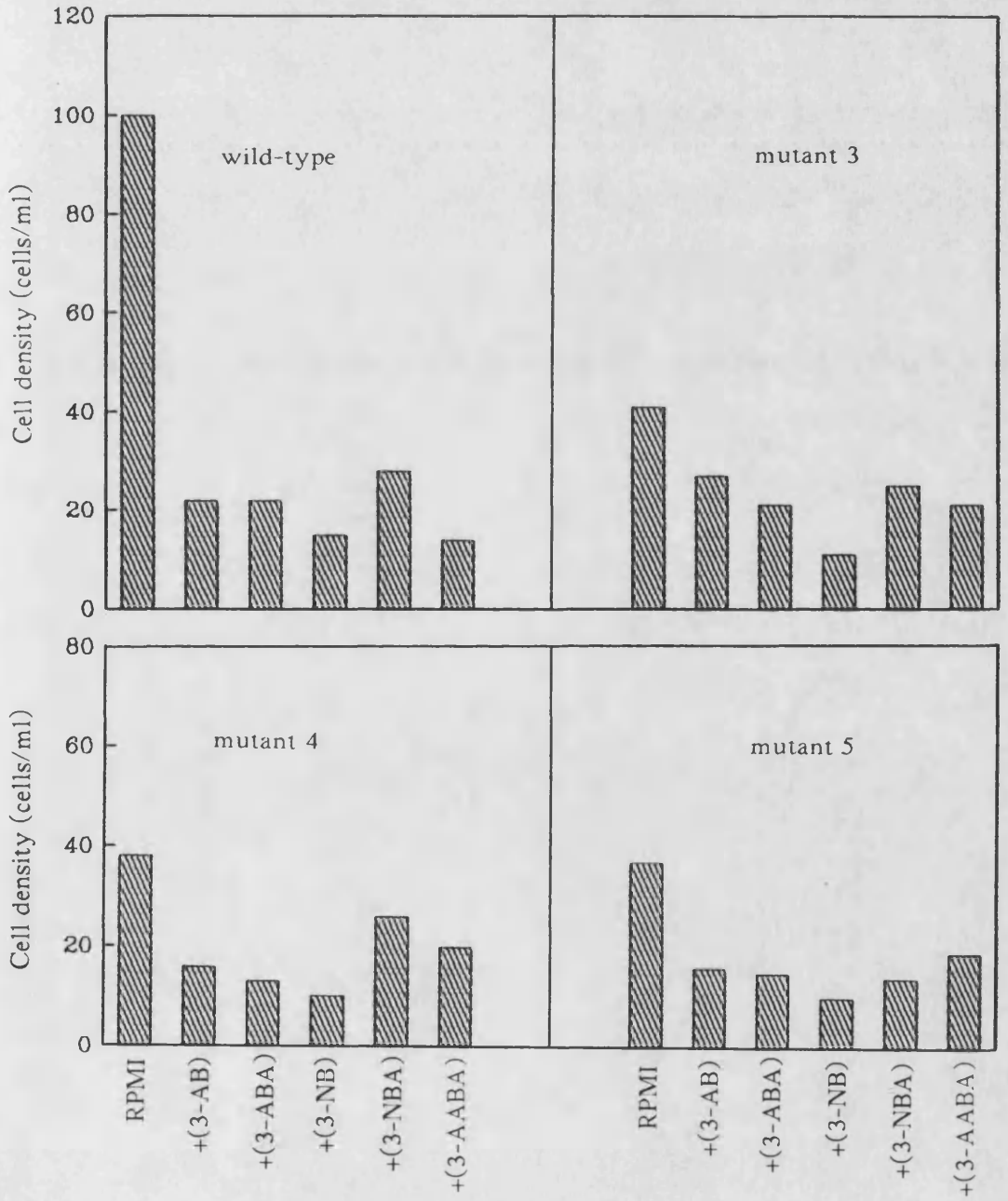


FIGURE 20.b Maximum cell density (cells/ml) at the stationary phase of mutants 6,7,9,10,12 and 13 cell lines in the presence of various compounds as described in figure 20.a.

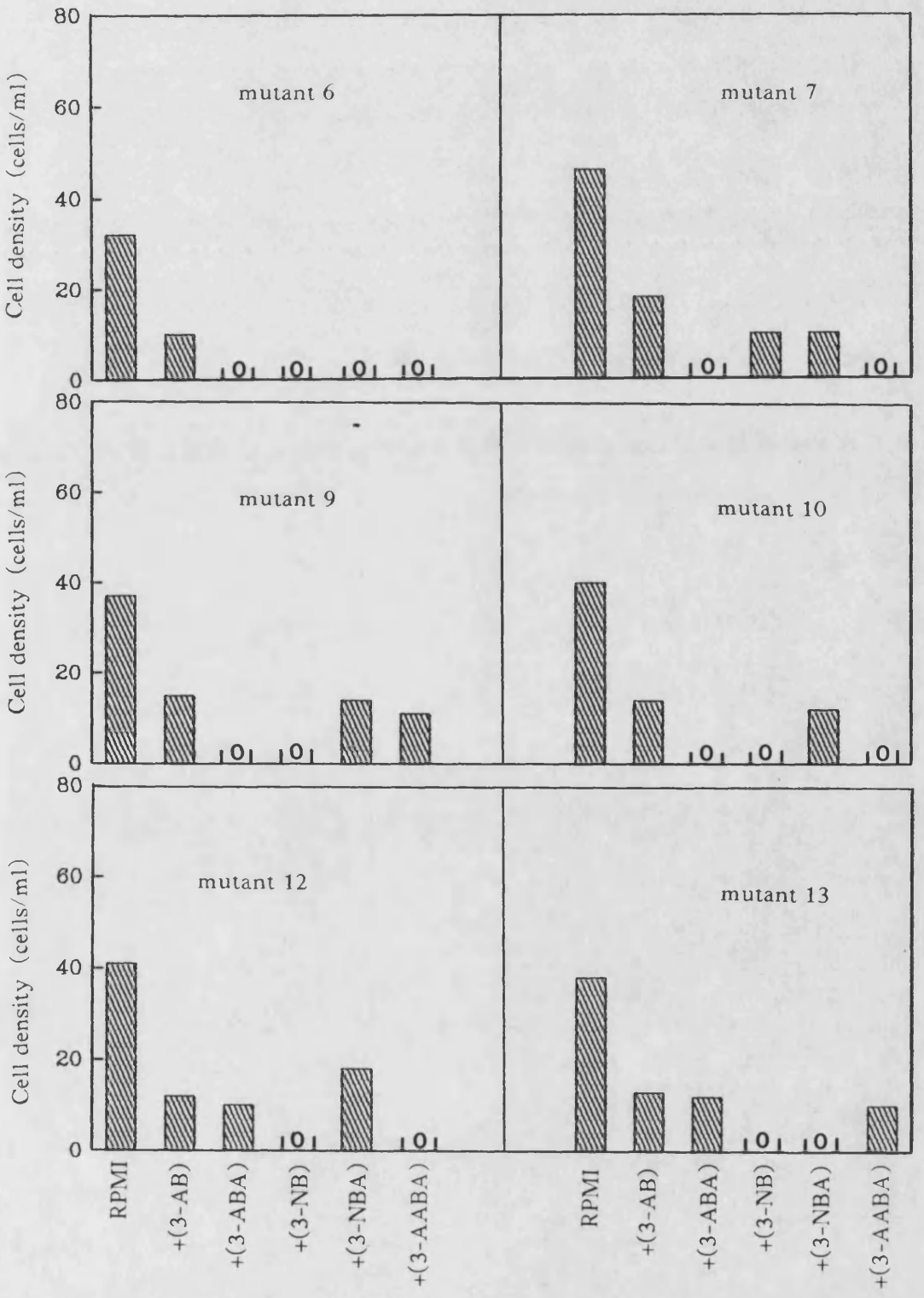
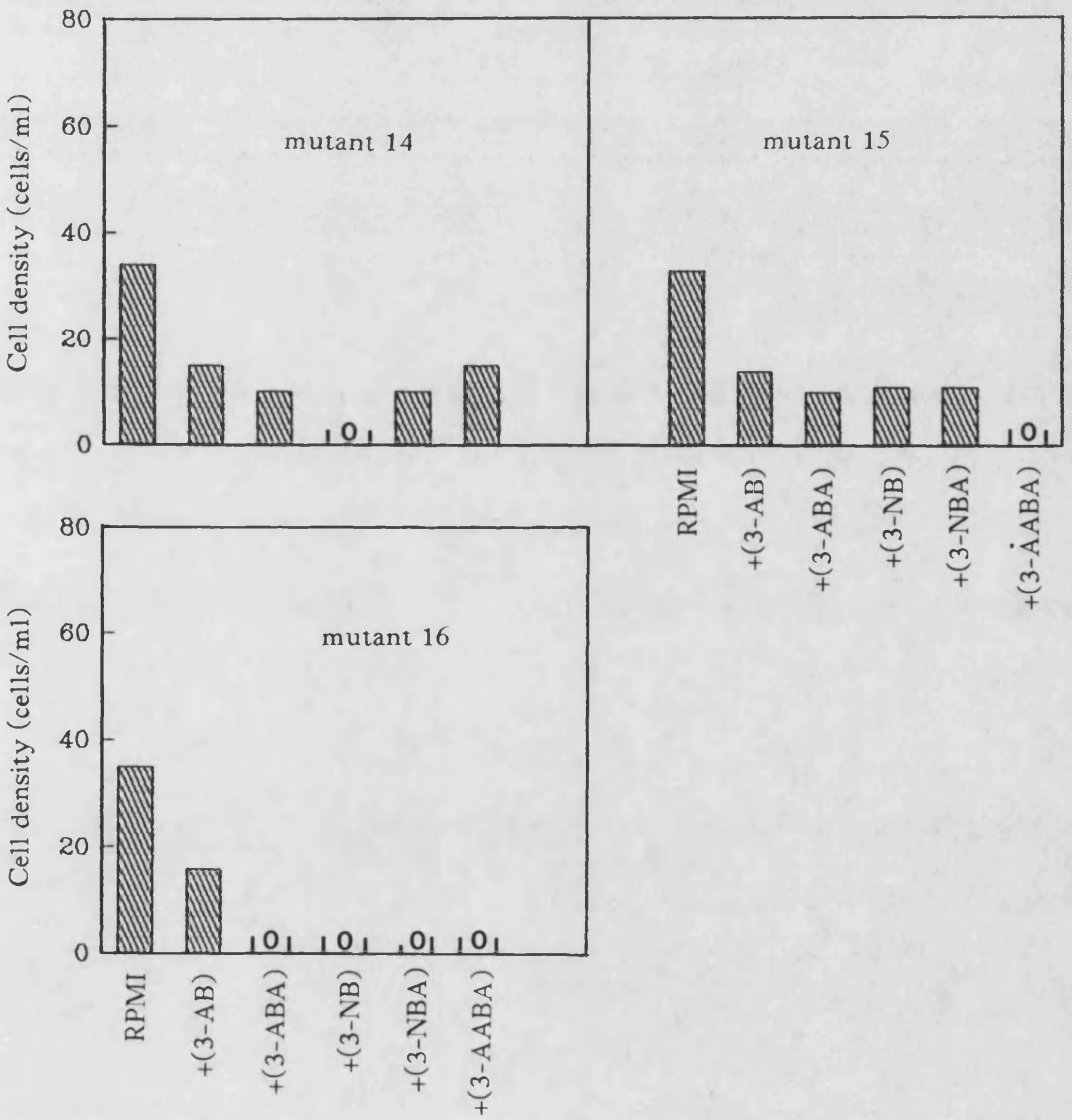


FIGURE 20.c Maximum cell density (cells/ml) at the stationary phase of mutants 14,15 and 16 cell lines in the presence of various compounds as deseribed in figure 20.a.



treatment.

DISCUSSION

This method of measuring "cell survival" in liquid culture has an advantage over estimation of "cell survival" assay of cloning in soft agar as the former method is simpler and more convinient to perform, results can be obtained within 3-4 days. The latter method is a more stringent test as it directly estimates the ability of the individual cells to repair damaged DNA or overcome the inhibitory effects of ADPR transferase inhibitors and resume normal division to form colonies but the process is slow and it requires at least 2-3 weeks to obtain the results.

This method of measuring the cytotoxicity of various compounds on mutant cell lines has shown that the cell cycles of mutant cell lines are affected by the ADPR transferase inhibitors and the acid analogues which resulted in the treated cells requiring longer times to undergo the process of cell division. Mutant 3 is the least sensitive whilst mutants 6 and 16 are the most sensitive towards 3-nitrobenzamide and all the acid analogues. Mutant cell lines that are sensitive to acid analogues (mutants 6, 7, 9, 10, 12, 13, 15 and 16) indicates that their target is not ADPR transferase but that they may interefere with other metabolic processes.

Results obtained for this investigation show that mutant cell lines are sensitive to 14 mM 3-aminobenzamide and 3 mM 3- nitrobenzamide (these concentrations are toxic to wild-type cells). Although mutant 3 is less sensitive to 14 mM 3-aminobenzamide and the 3 acid analogues, it is sensitive to 3mM 3-nitrobenzamide. This may be due to the combined effects of the nitro and the amide groups. The carboxylate ion of 3- nitrobenzoic acid at physiological pH may not contribute to

the cytotoxicity which resulted in the differences in the sensitivity of 3-nitrobenzamide and its acid analogue.

Mutant and wild-type cell lines were treated with high doses of compounds for long periods of time. The effect of cytotoxicity of ADPR transferase inhibitors on mutant and wild-type cell lines can not be solely based on the assumption that it results from the inhibition of poly (ADP-ribose) synthesis. This is due to recent reports emerging in the last few years which suggest that ADPR transferase inhibitors also affect other metabolic pathways. Cleaver *et al.*(1983) reported that 3-aminobenzamide (3-AB) inhibits the *de novo* pathway of purine biosynthesis as shown by the reduction of[^{14}C] label in adenine and guanine in HeLa cells. The same group (Milam and Cleaver, 1984) has further reported that 3-AB significantly inhibited the incorporation of [^{3}H] methionine and [^{14}C] glucose into DNA and that 3-aminobenzoic acid affects the salvage routes of DAN synthesis by reducing the [^{3}H] thymidine incorporation into DNA and glucose metabolism as measured by the rate of conversion of D-[^{14}C]-glucose to $^{14}CO_2$. The inhibition incorporation of [^{3}H] methyl group of methionine into deoxyguanosine, deoxyadenosine and particularly deoxycytidine by 3-AB was found to occur even at concentrations as low as 1 mM. Results also suggest that 3-AB affects the metabolism of 1 carbon pool which is essential for *de novo* purine and pyrimidine biosynthesis, especially on the pathway for *de novo* pyrimidine deoxynucleotide synthesis as it reduced the incorporation of radioactively labelled [^{3}H] glucose into pyrimidine to a greater extent than into purines. (Milam *et al.*, (1986). Similar results were also observed in human fibroblasts by Poirier *et al.* (1985). Schwartz *et al.* (1983) found that the rate of DNA synthesis in CHO cells is inhibited in the prolonged exposure of 3-aminobenzamide. Boyle (1985) suggests that 3-AB behaves as a general cell poison by inhibiting the incorporation of [^{14}C] formate into DNA, RNA and protein. 3-AB

was also reported to inhibit adenosine transport (Purnell *et al.*, 1985)

3-Nitrobenzamide is markedly more toxic to mutant cell lines compared to 3-aminobenzamide. Nitro-compounds have been shown to be cytotoxic and genotoxic which was reported to occur by the reduction to reactive intermediates which can then modify DNA (Biaglow 1981). It also affects molecular synthesis by inhibiting [^{3}H] uridine incorporation in RNA synthesis. (Kidwell *et al.*, 1985)

CONCLUSIONS

The resistance of mutant cells to 3-acetamidobenzamide (3-AAB) is dramatically greater than that of wild-type cells. If a defect in ADPR transferase were to be responsible for such resistance, it will have to be large. In the present series of experiments the only differences in the poly (ADP-ribose) metabolism of mutant and wild-type cell lines were minor. These differences could not account for the observed resistance. Results have shown that there is no difference in any of the following aspects.

1) Poly (ADP-ribose) synthesis.

2) NAD synthesis using [^{14}C] nicotinamide as precursor.

3) Response to DMS and its enhancement by 3-AAB.

4) The cytotoxicity of 3-aminobenzamide (3-AB) and 3- nitrobenzamide was equally effective on mutant and wild- type cell lines with the exception that mutant 3 has shown a higher resistance to 3-AB.

Mutant cells deficient in ADPR-transferase activity using 3-AAB as a selective agent in this thesis have not been found. The attempts for such mutant cells have also been explored in other laborateries using 5-methyl nicotinamide (Kidwell and Burdette, 1974; Nduka and Shall, 1980) and 3-aminobenzamide (Tavassoli *et al.*, 1987 b) as a selective agents. In some cases, cells were mutagenised first with alkylating agents and were then isolated with ADPR transferase inhibitor (Murray *et al.*, 1986), or with NAD starvation (Chatterjee *et al.*, 1987). Results obtained from the above investigations have shown that none of the mutant cell lines were deficient in ADPR transferase. Results shown in this thesis and others have

indicated that mutation in ADPR transferase genes is probably lethal to cells such that a deficiency in ADPR transferase would lead to a defect in vital functions resulting in cell death.

Although the mutant cell lines isolated in this thesis are not the type of mutant which was originally sought for, they may be of value in other aspects unrelated to ADP-ribosylation. It is possible that the effects of 3-AAB on other metabolic pathways as reported by various workers (see chapter 1.1.3) other than ADPR transferase may no longer exist in mutant cell lines, such that the cell cycle of G_1 and G_2 in mutant cells may no longer be affected by 3-AAB as reported by Kidwell *et al.* (1985). Mutant cell lines, especially mutant 3 which is shown to be resistant also to 3-AB, may be of use as a model cell to study the biological role of ADPR transferase using inhibitors as a tool such that the effect of ADPR transferase inhibitors (3- AAB or 3-AB) on mutant 3 may only select ADPR transferase as their target and not other metabolic pathways.

Further work should, therefore, be carried out on mutant 3 cells to ascertain that 3-AAB (at concentrations below 5 mM) does not interfere with DNA synthesis by investigation the incorporation of [^{3}H] methionine and [^{14}C] glucose into DNA (Milam and Cleaver 1984). The investigation of glycine incorporation into ATP and GTP and thymidine into DNA should also be carried out as their incorporation was shown to be inhibited by 3-AAB (at 1 mM) in L1210 and CHO cells (Hunting *et al.* 1985). If mutant 3 is shown not to be affected by 3-AAB this will provide the opportunity to study the biological role of ADPR transferase using 3-AAB as a tool in mutant 3 with the certainty that it only acts on ADPR transferase and does not perturb other metabolic pathways. Furthermore, the determination of Ki, the rate of up-take of [^{3}H] 3-AAB and DNA repair in mutant 3 should be useful in evaluating the genetic abnormality of these mutants towards the resistance of 3-AAB.

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